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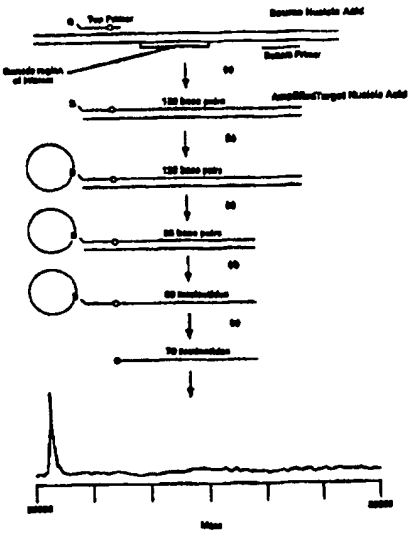
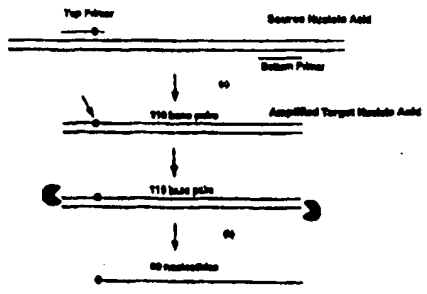
(54) Title: METHODS OF PREPARING NUCLEIC ACIDS FOR MASS SPECTROMETRIC ANALYSIS

(57) Abstract

This invention relates to methods for screening nucleic acids for polymorphisms by analyzing amplified target nucleic acids using mass spectrometric techniques and to procedures for improving mass resolution and mass accuracy of these methods of detecting polymorphisms.





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DESCRIPTION

METHODS OF PREPARING NUCLEIC ACIDS FOR MASS SPECTROMETRIC ANALYSIS

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CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. Application Serial No. 60/032,369 filed December 2, 1996 and U.S. Application Serial No. 08/759,993 filed December 2, 1996 which is a continuation-in-part of U.S. Application Serial No. 08/715,582 filed September 19, 1996.

INTRODUCTION

Approximately 4,000 human disorders are attributed to genetic causes. Hundreds of genes responsible for various disorders have been mapped, and sequence information is being accumulated rapidly. A principal goal of the Human Genome Project is to find all genes associated with each disorder. The definitive diagnostic test for any specific genetic disease (or predisposition to disease) will be the identification of polymorphic variations in the DNA sequence of affected cells that result in alterations of gene function. Furthermore, response to specific medications may depend on the presence of polymorphisms. Developing DNA (or RNA) screening as a practical tool for medical diagnostics requires a method that is inexpensive, accurate, expeditious, and robust.

Genetic polymorphisms and mutations can manifest themselves in several forms, such as point polymorphisms or point mutations where a single base is changed to one of the three other bases, deletions where one or more bases are removed from a nucleic acid sequence and the bases flanking the deleted sequence are directly linked to each other, insertions where new bases are inserted at a particular point in a nucleic acid sequence adding additional length to the overall sequence, and expansions and reductions of repeating sequence motifs. Large insertions and deletions, often the result of chromosomal recombination and rearrangement events, can lead to

partial or complete loss of a gene. Of these forms of polymorphism, in general the most difficult type of change to screen for and detect is the point polymorphism because it represents the smallest degree of molecular change.

Although a number of genetic defects can be linked to a specific single point mutation within a gene, *e.g.* sickle cell anemia, many are caused by a wide spectrum of different mutations throughout the gene. A typical gene that might be screened could be anywhere from 1,000 to 100,000 bases in length, though smaller and larger genes do exist. Of that amount of DNA, only a fraction of the base pairs actually encode the protein. These discontinuous protein coding regions are called exons and the remainder of the gene is referred to as introns. Of these two types of regions, exons often contain the most important sequences to be screened. Several complex procedures have been developed for scanning genes in order to detect polymorphisms. These procedures are applicable to both exons and introns.

In terms of current use, most of the methods to scan or screen genes employ slab or capillary gel electrophoresis for the separation and detection step in the assays. Gel electrophoresis of nucleic acids primarily provides relative size information based on mobility through the gel matrix. If calibration standards are employed, gel electrophoresis can be used to measure absolute and relative molecular weights of large biomolecules with some moderate degree of accuracy; even then typically the accuracy is only 5% to 10%. Also the molecular weight resolution is limited. In cases where two DNA fragments with the identical number of base pairs can be separated, using high concentration polyacrylamide gels, it is still not possible to identify which band on a gel corresponds to which DNA fragment without performing secondary labeling experiments. Thus, gel electrophoresis techniques can only determine size and cannot provide any information about changes in base composition or sequence without performing more complex sequencing reactions. Gel-based techniques, for the most part, are dependent on labeling or staining methods to visualize and discriminate between different nucleic acid fragments.

All of the methods in use today capable of screening broadly for genetic polymorphisms suffer from technical complication and are labor and time intensive. Single strand conformational polymorphism (SSCP) (Orita *et al.*, 1989), denaturing gradient gel electrophoresis (DGGE) (Abrams *et al.*, 1990), chemical cleavage at mismatch (CCM) (Saleeba and Cotton, 1993), enzymatic mismatch cleavage (EMC) (Youil *et al.*, 1995), and "cleavase" fragment length polymorphism (CFLP) procedures are currently gel-based, making them

cumbersome to automate and perform efficiently. There is a need for new methods that can provide cost effective and expeditious means for screening genetic material in an effort to reduce medical expenses.

The late 1980's saw the rise of two new mass spectrometric techniques for successfully measuring the masses of intact very large biomolecules, namely, matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry (TOF MS) (Tanaka *et al.*, 1988; Spengler *et al.*, 1989) and electrospray ionization (ESI) combined with a variety of mass analyzers (Fenn *et al.*, 1989). Both of these methods are suitable for genetic screening tests. The MALDI mass spectrometric technique can also be used with methods other than time-of-flight, for example, magnetic sector, Fourier-transform ion cyclotron resonance, quadrupole, and quadrupole trap. One of the advances in MALDI analysis of polynucleotides was the discovery of 3-hydroxypicolinic acid ("3-HPA") as a matrix for mixed-base oligonucleotides (Wu, *et al.*, 1993).

MALDI-TOF MS involves laser pulses focused on a small sample plate comprising analyte molecules (*i.e.* nucleic acids) embedded in either a solid or liquid matrix which is typically a small, highly absorbing material. The laser pulses transfer energy to the matrix causing a microscopic ablation and concomitant ionization of the analyte molecules, producing a gaseous plume of intact, charged nucleic acids in single-stranded form. If double-stranded nucleic acids are analyzed, the MALDI-TOF MS typically results in mostly denatured single-strand detection. The ions generated by the laser pulses are accelerated to a fixed kinetic energy by a strong electric field and then pass through an electric field-free region in vacuum, traveling with a velocity corresponding to their respective mass-to-charge ratios (m/z). Thus, the smaller m/z ions will travel through the vacuum region faster than the larger m/z ions thereby causing a separation. At the end of the electric field-free region, the ions collide with a detector that generates a signal as each set of ions of a particular mass-to-charge ratio strikes the detector. Usually for a given assay, 10 to 100 mass spectra resulting from individual laser pulses are summed together to make a single composite mass spectrum with an improved signal-to-noise ratio.

The mass of an ion (such as a charged nucleic acid) is measured by using its velocity to determine the mass-to-charge ratio by time-of-flight analysis. In other words, the mass of the molecule directly correlates with the time it takes to travel from the sample plate to the detector. The entire process takes only microseconds. In an automated apparatus, tens to hundreds of

samples can be analyzed per minute. In addition to speed, MALDI-TOF MS has one of the largest mass ranges for mass spectrometric devices. The current mass range for MALDI-TOF MS is from 1 to 1,000,000 Daltons (Da) (measured recently for a protein) (Nelson *et al.*, 1995).

The performance of a mass spectrometer is measured by its sensitivity, mass resolution and mass accuracy. Sensitivity is measured by the amount of material needed; it is generally desirable and possible with mass spectrometry to work with sample amounts in the femtomole and low picomole range. Mass resolution, $m/\Delta m$, is the measure of an instrument's ability to produce separate signals from ions of similar mass. Mass resolution is defined as the mass, m, of an ion signal divided by the full width of the signal, Δm, usually measured between points of half-maximum intensity. Mass accuracy is the measure of error in designating a mass to an ion signal. The mass accuracy is defined as the ratio of the mass assignment error divided by the mass of the ion and can be represented as a percentage.

To be able to detect any point polymorphism directly by MALDI-TOF mass spectrometry, one would need to resolve and accurately measure the masses of nucleic acids in which a single base change has occurred (in comparison to the wild type nucleic acid). A single base change can be a mass difference of as little as 9 Da. This value represents the difference between the two bases with the closest mass values, A and T (A = 2'-deoxyadenosine-5'-phosphate = 313.19 Da; T = 2'-deoxythymidine-5'-phosphate = 304.20 Da; G =2'-deoxyguanosine-5'-phosphate = 329.21 Da; and C = 2'-deoxycytidine-5'-phosphate = 289.19 Da). If during the mutation process, a single A changes to T or a single T to A, the mutant nucleic acid containing the base transversion will either decrease or increase by 9 Da in total mass as compared to the wild type nucleic acid. For mass spectrometry to directly detect these transversions, it must therefore be able to detect a minimum mass change, Δm, of approximately 9 Da.

For example, in order to fully resolve (which may not be necessary) a point-mutated (A to T or T to A) heterozygote 50-base single-stranded DNA fragment having a mass, m, of ~ 15,000 Da from its corresponding wild type nucleic acid, the required mass resolution is $m/\Delta m = 15{,}000/9 \approx 1{,}700$. However, the mass accuracy needs to be significantly better than 9 Da to increase quality assurance and to prevent ambiguities where the measured mass value is near the half-way point between the two theoretical masses. For an analyte of 15,000 Da, in practice the mass accuracy needs to be $\Delta m \sim \pm 3$ Da = 6 Da. In this case, the absolute mass accuracy required is (6/15,000)*100 = 0.04%. Often a distinguishing level of mass accuracy relative to another

known peak in the spectrum is sufficient to resolve ambiguities. For example, if there is a known mass peak 1000 Da from the mass peak in question, the relative position of the unknown to the known peak may be known with greater accuracy than that provided by an absolute, previous calibration of the mass spectrometer.

In order for mass spectrometry to be a useful tool for screening for polymorphisms in nucleic acids, several basic requirements should be met. First, any nucleic acids to be analyzed should be purified to minimize the presence of salt ions and other molecular contaminants. These impurities may reduce the intensity and quality of the mass spectrometric signal to a point where either (i) the signal is undetectable or unreliable, or (ii) the mass accuracy and/or resolution is below the value necessary to detect the type of polymorphism expected. Second, the size of the nucleic acids to be analyzed should be within the range where there is sufficient mass resolution and accuracy. Mass accuracy and resolution significantly degrade as the mass of the analyte increases. Currently, the detection of single nucleotide polymorphisms (SNPs) above said mass value is difficult above a mass of approximately 30,000 Da for oligonucleotides (~ 100 bases). Third, because all molecules within a sample are visualized during mass spectrometric analysis (i.e. it is not possible to selectively label and visualize certain molecules and not others as one can with gel electrophoresis methods), nucleic acid samples should be partitioned prior to analysis to remove unwanted nucleic acid products from the spectrum. Fourth, the mass spectrometric methods for generalized nucleic acid screening must be efficient and cost effective in order to screen a large number of nucleic acid bases in as few steps as possible.

The methods for detecting nucleic acid polymorphisms known in the art do not satisfy these four requirements. For example, current methods for mass spectrometric analysis of DNA fragments have focused on double-stranded DNA fragments which result in complicated mass spectra, making it difficult to resolve mass differences between two complementary strands (see, *e.g.*, Tang *et al.*, 1994). Thus, there is a need for cost and time effective methods of detecting genetic polymorphisms using mass spectrometry, preferably MALDI or ESI, and with mass accuracy of a few parts in 10,000 or better.

SUMMARY OF THE INVENTION

This invention provides novel methods and kits for the screening of target nucleic acids and the identification of changes in base composition that might result from a genetic polymorphism. The present invention discloses novel processes focusing on the use of mass

spectrometry as a genetic analysis tool and employing the unique properties of mass spectrometry and MALDI-TOF MS, in particular, to separate different amplified single-stranded target nucleic acids and identify their mass exactly. Significantly, mass spectrometry requires only minute samples, provides extremely detailed information about the molecules being analyzed including high mass accuracy, and is easily automated.

The present invention encompasses several embodiments, such as (1) procedures for reducing the length of target nucleic acids by removing one or more flanking polynucleotide regions that "flank," or are adjacent to or near, the regions of interest; (2) procedures for isolating either single-stranded or double-stranded target nucleic acids for mass spectrometric analysis; (3) procedures combining these two aspects; and (4) kits for the methods described herein.

The present invention encompasses several embodiments, such as (1) procedures for preparing a double-stranded target nucleic acid for mass spectrometric analysis; (2) procedures for determining the mass of target nucleic acids, where the target nucleic acid may be either single-stranded or double-stranded; and (3) kits for preparing a double-stranded target nucleic acid for mass spectrometric analysis. It will be understood by those of skill in the art that where the nucleic acid is double-stranded, the two strands are complementary to each other and are connected via hydrogen bonds along the strands.

An embodiment of the present invention encompasses a method of determining the mass of a target nucleic acid by mass spectrometric analysis. This method generally includes: identifying a target nucleic acid; reducing the length of the target nucleic acid by cleaving at least a portion of one or more of the flanking regions to produce a reduced-length target nucleic acid; obtaining a single-stranded reduced-length target nucleic acid; and determining the mass of the single-stranded reduced-length target nucleic acid using a mass spectrometer. Typically, the target nucleic acid will contain a region of interest and one or more flanking regions.

A preferred embodiment encompasses amplifying the target nucleic acid prior to reducing the length of the target nucleic acid to produce an amplified target nucleic acid. The amplified target nucleic acid may be subsequently reduced in length and obtained in single-stranded form, free of its complement, for mass spectral analysis. The target nucleic acid may be amplified by any method known by one of skill in the art, for example, polymerase chain reaction ("PCR™", with PCR™ being a preferred amplification method. These methods are well known by those of skill in the art.

It is contemplated that one of skill in the art may use the methods of this invention to analyze more than one target nucleic acid simultaneously. As used herein "a" will be understood to mean one or more. Thus, "a target nucleic acid" may refer, for example, to one, two, three, four, five or more target nucleic acids. Aspects of this invention, therefore, include determining the mass of one single-stranded reduced-length target nucleic acid as well as determining the masses of multiple single-stranded reduced-length target nucleic acids simultaneously or in seriatim. Where the masses of multiple single-stranded reduced-length target nucleic acids are being determined, each of the target nucleic acids may be reduced in length by the same or a different method. Similarly, the single-stranded reduced-length target nucleic acids may be obtained from the reduced-length target nucleic acids by the same or different methods. For example, if two target nucleic acids are identified, or selected, for analysis, then these two target nucleic acids may both be reduced in length by an endonuclease, or one may be reduced in length by an endonuclease and the other by cleaving at a chemically cleavable site, and so on.

The target nucleic acids encompassed by this invention will generally contain a region of interest and one or more flanking regions. A "region of interest" refers to the region for which one is interested in determining the mass. For example, when the methods disclosed in this invention are employed to detect or screen for polymorphisms, the region of interest would be the region containing, or that is suspected of containing, a polymorphism. The flanking regions are the portions of DNA sequence on either side of the region of interest.

For embodiments employing PCR™ primers and polymerases to amplify a target nucleic acid, the primer is often complementary to a portion of one or more flanking regions of the target nucleic acid to allow the primer to effectively anneal to the target nucleic acid and provide a site to extend a complement to the target nucleic acid via PCR™. Therefore, for the methods comprising amplification, it is preferred that at least one of the primers is complementary to a portion of a flanking region that is preferably adjacent to or close to the polynucleotide region of interest, generally within 40 nucleotides.

When the methods of this invention are used to detect a polymorphism, the target nucleic acids employed in this invention may include any polynucleotide sequence that contains or is suspected of containing a polymorphism, including but not limited to short tandem repeats (STRs), simple sequence length polymorphisms (SSLP), single nucleotide polymorphisms (SNPs), and any of a multitude of disease markers, for example, markers for sickle cell anemia,

fragile X disorder, cystic fibrosis, Tay Sachs disease, Gaucher disease, thalassemias, and cancer-related genes. While the target nucleic acids for use in conjunction with the present invention may be double- or single-stranded, it is preferable that the nucleic acids be obtained in single-stranded form, free of its complementary strand prior to MS analysis. These single-stranded target nucleic acids may be any size that can be adequately resolved by mass spectrometric analysis. Preferably, in cases where a SNP is to be detected, the final product single-stranded amplified target nucleic acids are less than about 100 bases in length. More preferably, the final product, single-stranded amplified target nucleic acids are from about 10 to 90 bases in length. As used in this context, "about" means anywhere from ± 1 to 10 base pairs, and all the integers in between, for example, ±1, ±2, ±3, ±4, ±5, ±6, ±7, ±8, ±9, or ±10 base pairs.

However, one of ordinary skill in the art will appreciate that as mass spectrometric techniques for analysis of nucleic acids improve, the sizes of single-stranded amplified target nucleic acids useful in this invention can be increased. The nature of the mutation to be detected is also a factor in the size limitations for optimum mass resolution. For example, as described above for SNPs, the maximum size limit may be approximately 100 nucleotides in length. However, for microsatellite repeats and other two nucleotide repeats, the maximum size limit may be approximately 200 nucleotides in length, and the maximum size limit for four-nucleotide repeats may be approximately 300 nucleotides.

The target nucleic acids of this invention may be either double-stranded or single-stranded. As used herein, the phrase "obtaining a single-stranded reduced-length target nucleic acid" refers to isolating a single-stranded nucleic acid free from its complement for purposes of mass spectral analysis. Where the target nucleic acid is single-stranded, it will be understood by those of skill in the art that no further steps are required to obtain the single-stranded reduced-length target nucleic acid from the reduced-length target nucleic acid. However, where the target nucleic acid is double-stranded, one of the two complementary strands must be separated or isolated from the other such that only one of the two strands is subjected to mass spectrometry, *e.g.*, by binding one of the strands to a solid support, denaturing the double-stranded nucleic acid and isolating either the bound or unbound strand free from its complement. This allows for greater mass resolution, simplifies the spectrum, and eliminates the collection of cumulative information.

The term complementary refers to the formation of sufficient hydrogen bonding between two nucleic acids to stabilize a double-stranded nucleotide sequence formed by hybridization of the two nucleic acids.

The methods for reducing the length of target nucleic acids eliminate unnecessary sequences and reduce the mass of the resulting single-stranded or double-stranded target nucleic acids, resulting in increased mass resolution and accuracy.

Exemplary methods of reducing length include: cleaving at endogenous restriction endonuclease cleavable sites present in one or more flanking regions but absent in the region of interest; cleaving at restriction endonuclease cleavable sites which are at or adjacent to restriction endonuclease recognition sites incorporated into one or more of the flanking regions where the cleavable sites are introduced into the flanking regions using of one or more cleavable primers containing restriction endonuclease recognition sites within their sequences; cleaving at a combination of restriction endonuclease cleavable sites where the sites are endogenous and/or introduced using mismatch or overhanging primers; selective digestion of one or more flanking regions using exonuclease and an exonuclease blocking moiety to protect the regions of interest from digestion; and chemically cleaving at a chemically cleavable site. For embodiments where cleavable sites are employed, the cleavable sites are often located in or near a flanking region. However, the target nucleic acids may be reduced in length by any of the methods known by those of skill in the art for cleaving within one or more flanking regions preferably without cleaving within the region of interest.

Another aspect of the invention involves the use of cleavable primers to reduce the length of an amplified target nucleic acid. An amplified target nucleic acid may be reduced in length by cleaving at least a portion of one or more of the flanking regions having a cleavable site. In this context, the cleavable site may be introduced via a cleavable primer and may be located outside of the region of interest. Cleavable primers of the invention may include those having an exonuclease blocking moiety, a Type IIS restriction endonuclease recognition site, a Type II restriction endonuclease recognition site, and sites capable of being chemically cleaved.

The restriction endonucleases employed with the present invention may include type II and type IIS restriction endonucleases. The restriction endonuclease recognition sites may be either within a primer region, or outside the primer region, so long as the restriction endonuclease cleavable sites are within or near one or more of the flanking regions. The restriction endonuclease recognition sites are preferably not within a region of interest. For type

II restriction endonucleases, the restriction endonuclease recognition site is the same as the restriction endonuclease cleavable site. For Type IIS restriction endonucleases, the cleavable site is at a defined distance away from one side of the recognition site, usually from about 14 to about 20 base pairs away. Thus, if the Type IIS recognition site is contained within a flanking region, the endonuclease cleaving site must be within about 20 bases of that flanking region and is preferably within 14 about bases of that flanking region. Thus, the term "near" as employed in this aspect of the invention means "within about 20 bases."

Another embodiment of the invention involves reducing the length of an amplified target nucleic acid and isolating a single-stranded amplified target nucleic acid at the same time by using a cleavable primer having an exonuclease blocking moiety. After amplification of the target nucleic acid, the amplified target nucleic acid will include an exonuclease blocking moiety. The amplified target nucleic acid is then treated with a 5' to 3' exonuclease, which degrades the strand containing the exonuclease blocking moiety in a 5' to 3' direction only up to the blocking moiety. The 5' to 3' exonuclease may optionally degrade the other complementary strand of the amplified target nucleic acid, in cases where the other strand does not have an exonuclease blocking moiety. The treatment with the 5' to 3' exonuclease leaves a reduced-length, single-stranded amplified target nucleic acid for mass spectrometric analysis.

Cleavable sites within cleavable primers may include chemically cleavable groups incorporated within the phosphate backbone linkage (*e.g.* replacement of phosphate with a phosphoramidate) or as a substituent on or replacement of one of the bases or sugars of the oligonucleotide primer (*e.g.* a modified base or sugar, for example, a more labile glycosidic linkage). Such chemically cleavable groups would be apparent to one of skill in the art in light of the present disclosure and include, for example, dialkoxysilane, 3'-(S)-phosphorothioate, 5'-(S)-phosphorothioate, 3'-(N)-phosphoroamidate, 5'-(N)-phosphoroamidate, and ribose. FIGS. 16A and 16B depict a 3'-(S)-phosphorothioate and 5'-(S)-phosphorothioate, respectively as defined in this invention. Note that these linkages are often referred to as thiophosphates as well. A similar nomenclature is employed for 3'-(N)-phosphoroamidate, 5'-(N)-phosphoroamidate. The chemically cleavable site should generally be stable under the amplification, hybridization and washing conditions to be employed and is preferably within one or more of the flanking regions.

In a preferred embodiment, the cleavable site is located near the 3' end of the primer used to bind the amplified target nucleic acid to the solid support. By locating the cleavable site near

the 3' end, it is possible to further reduce the length of the amplified target nucleic acid, eliminating a flanking region from the polynucleotide region of interest. Cleavable primers are described in PCT/US96/06116, filed April 26, 1996 (incorporated herein by reference).

Accordingly, cleavable primers may contain one or more restriction recognition sites of one or more different restriction endonucleases; one or more cleavable sites of one or more different restriction endonucleases; one or more exonuclease blocking moieties; one or more sites capable of chemical cleavage; or a combination thereof.

The present invention also provides methods for obtaining single-stranded or double-stranded amplified target nucleic acids. The isolation methods include direct attachment of one of the two strands of a double-stranded amplified target nucleic acid or a set of such molecules, to a solid support. The isolation methods further include indirect attachment of a single-stranded or double-stranded amplified target nucleic acid, or a set thereof, to a solid support via an attachment capable of attaching to a solid support via covalent or noncovalent attachment. Methods of direct attachment include for example, biotin/avidin interactions, as well as other methods known by those of skill in the art.

For example, in one embodiment, a strand of an amplified target nucleic acid may be bound or attached to a solid support to permit rigorous washing and concomitant removal of salt adducts, unwanted oligonucleotides and enzymes. Either a double-stranded amplified target nucleic acid or a single-stranded amplified target nucleic acid may be isolated for mass spectrometric analysis. The single-stranded amplified target nucleic acid analyzed by MS may be either the strand bound or not bound to the solid support.

When the unbound strand is used for MS analysis, it is typically purified by first washing the bound strand and its attached complement under conditions not sufficiently rigorous to disrupt the strand's attachment to its bound complement. After unwanted biomolecules and salts are removed, the complement may then be released under more rigorous conditions (see FIG. 11).

In contrast, when the bound strand is to be analyzed, it is typically washed under more vigorous conditions such that the interactions between the bound strand and its unbound complement is disrupted. This allows the unbound strand to be washed away with the other salts and unwanted biomolecules. Cleavable linkers or cleavable primers may be used to release the bound strand from the solid support prior to MS analysis.

The isolation methods described herein provide significantly improved mass resolution and accuracy in large mass ranges. Such isolation of either single-stranded or double-stranded amplified target nucleic acids generally occurs prior to the application of the nucleic acids to the matrix solution, resulting in well-defined mass spectral peaks and enhanced mass accuracy. The matrix solution can be any of the known matrix solutions used for mass spectrometric analysis, including 3-hydroxypicolinic acid ("3-HPA"), nicotinic acid, picolinic acid, 2,5-dihydroxybenzoic acid, and nitrophenol.

The reducing and obtaining steps may occur consecutively in any order or simultaneously. Thus, this invention encompasses (i) reducing the length of a target nucleic acid prior to isolating the single-stranded reduced-length target nucleic acid from its complement acid to obtain the single-stranded reduced-length target nucleic acid; (ii) isolating a single-strand of the full-length target nucleic acid free from its complement and then reducing the length of the single-stranded target nucleic acid to obtain the single-stranded reduced-length target nucleic acid; (iii) simultaneously reducing the length of the target nucleic acid and isolating it free from its complementary strand acid to obtain the single-stranded reduced-length target nucleic acid; or (iv) any combination of the above steps so long as acid a single-stranded reduced-length target nucleic acid is obtained free of its complementary strand prior to mass spectral analysis.

Another aspect of this invention encompasses a method of determining the mass of a target nucleic acid, where the target nucleic acid generally contains a first strand and a second complementary strand. The method of the invention includes: identifying a target nucleic acid; amplifying the target nucleic acid prior to reducing the length of the target nucleic acid to produce an amplified target nucleic acid; reducing the length of the target nucleic acid by cleaving at least a portion of one or more of the flanking regions to produce a reduced-length target nucleic acid; obtaining a single-stranded reduced-length target nucleic acid; and determining the mass of the single-stranded reduced-length target nucleic acid using a mass spectrometer where the target nucleic acid further comprises a region of interest and one or more flanking regions and where the obtaining step comprises attaching the first strand of the amplified target nucleic acid to a solid support and separating the first strand from the second strand to produce a bound first strand and an unbound second strand. In this embodiment, the mass of the unbound second strand is determined.

The present invention additionally encompasses primers and methods for using primers that are capable of being "attached" or bound to a solid support. Generally, this is accomplished

by attaching a binding group or moiety to the primer or to a modified nucleotide during amplification, where the binding group or moiety is capable of attaching or binding the oligonucleotide to the solid support. This binding moiety may be attached to the oligonucleotide primer or amplification product either directly, through an intervening linking group or by specific hybridization to an intermediary oligonucleotide which is itself bound to a solid support. Binding moieties include functional groups for covalent bonding to a solid support, ligands that attach to the solid support via a high-affinity, noncovalent interaction (such as biotin with streptavidin), a series of bases complementary to an intermediary oligonucleotide which is itself attached to the solid support, as well as other means that are well-known to those of skill in the art, such as those described in PCT WO 96/37630, incorporated herein by reference.

The first strand is typically separated from the second strand by washing under conditions rigorous enough to disrupt the double-stranded base pairing structure, but not rigorous enough to disrupt the attachment of the bound first strand to the solid support. The solution-phase (or washings) containing the unbound strand can then be prepared for mass spectral analysis.

Cleavable primers and sites as discussed above are also employed in this embodiment. However, the cleavable site should preferably not be between the binding moiety, i.e. the group attaching the first bound strand to the solid support, and the region of interest. Alternatively, the cleavable site should be incorporated into the second strand only, and not into the first strand that is to be attached to the solid support.

A preferred embodiment encompasses the use of a cleavable primer having a chemically cleavable group of 3'-(S)-phosphorothioate or 5'-(S)-phosphorothioate, where the first strand is biotinylated and bound to a solid support via a biotin:avidin interaction (*i.e.* where streptavidin beads are used for a solid support). It is also preferable to employ mass-modified nucleotides with this aspect of the invention.

Alternatively, the obtaining step may include (a) attaching the first strand of the amplified target nucleic acid to a solid support, (b) separating the first strand from the second strand to produce a bound first strand and an unbound second strand, (c) removing the unbound second strand, and (d) releasing the bound first strand from the solid support to produce a single-stranded reduced-length amplified target nucleic acid for mass spectral analysis. In this embodiment, the mass of the bound first strand is determined using a mass spectrometer.

Several methods may be employed to release the reduced-length single-stranded amplified target nucleic acid from the solid support. Generally, the methods used must either

employ reversible chemical interactions between the binding group and the solid support, that is, a "cleavable linker," or a separate chemically or enzymatically cleavable site somewhere within the bound product. Thus, these methods for releasing the bound strand include all of the methods that may be used for reducing the length of the bound strand as well. For example, an exonuclease blocking group, endonuclease recognition site, or a chemically cleavable site may be incorporated into the bound strand between the binding moiety and the region of interest, cleaving at one of these sites through use of an exonuclease, endonuclease, or a chemical agent accomplishes both the releasing and the reduction in length simultaneously. When more than one target nucleic acid is identified for analysis, the target nucleic acids may be released and analyzed at the same time or consecutively.

This invention also encompasses methods for release that do not include reducing the length of the amplified or unamplified target nucleic acids depending on the method used to bind the amplified target nucleic acid to the solid support. For example, both the hybridization and biotin/streptavidin methods employ means such as denaturation to disrupt the noncovalent interactions and cause the release of the bound single-stranded target nucleic acids. It may be preferred to use a chemically cleavable site with the biotin/streptavidin method so that release of the target nucleic acids can be performed under relatively mild conditions.

Another embodiment of this invention encompasses a method of preparing a double-stranded target nucleic acid for mass spectrometric analysis. This method generally includes comprising: amplifying a target nucleic acid to produce an amplified target nucleic acid; attaching the first strand of the amplified target nucleic acid to a solid support to produce a bound first strand and an unbound second strand; removing, or detaching, the unbound second strand from the bound first strand; releasing the bound first strand from the solid support to form a single-stranded amplified target nucleic acid; and determining the mass of the single-stranded amplified target nucleic acid using a mass spectrometer where the amplified target nucleic acid comprises a first strand and a second complementary strand. In this embodiment, the unbound second strand is typically removed from the bound first strand by denaturing and washing.

A preferred embodiment encompasses employing a cleavable linker during the releasing step, wherein the determining step preferably does not involve sequencing the amplified target nucleic acid.

The present invention also provides methods of detecting polymorphisms in one or more target nucleic acids. This embodiment generally includes: amplifying at least one target nucleic

acid; reducing the length of at least one of the amplified target nucleic acids comprising cleaving off a portion of one or more flanking regions, and determining the masses of each of the reduced-length amplified target nucleic acids using a mass spectrometer wherein said amplified target nucleic acid comprises a region of interest and one or more flanking regions. This method may be used to detect polymorphisms in a single target nucleic acid compared to a wild type target nucleic acid by detecting variability in mass. Other "alleles" of the target nucleic acid may also be detected using the methods of the invention.

In the present disclosure, "wild type" is the standard or reference nucleotide sequence to which variations are compared. Thus, by definition, any variation from wild type is considered a polymorphism, including naturally occurring sequence variations and pathogenic mutations.

In another embodiment, methods are provided for detecting polymorphisms in at least one target nucleic acid. These methods may include: amplifying at least one target nucleic acid; isolating either a positive or negative strand of the amplified target nucleic acid to form a single-stranded amplified target nucleic acid; and determining the masses of each single-stranded amplified target nucleic acid using a mass spectrometer where the amplified target nucleic acid comprises a region of interest and one or more flanking regions.

In yet another embodiment, methods are provided for detecting polymorphisms in at least one target nucleic acid by amplifying at least one target nucleic acid; reducing the length of at least one of the amplified target nucleic acids comprising cleaving off a portion of one or more flanking regions; isolating either a positive or negative strand of said amplified target nucleic acid to form an amplified target nucleic acid; and determining the mass of each single-stranded amplified target nucleic acid using a mass spectrometer where the amplified target nucleic acid comprises a region of interest and optionally one or more flanking regions

The methods described in the present invention may also be used to detect polymorphisms in a set of different target nucleic acids. In this context, the methods should generally include: amplifying each of the target nucleic acids; reducing the length and/or isolating a single-strand of each of said amplified target nucleic acids; and determining the mass of each of the single-strands of said amplified target nucleic acids using mass spectrometry. Thus, these methods can be used to detect polymorphisms in a plurality of different target nucleic acids simultaneously.

Using the methods described herein, one can uniquely identify a genomic sample by amplifying the target nucleic acids; isolating single-stranded amplified target nucleic acids; and

determining the masses of the single-stranded amplified target nucleic acids using mass spectrometry. The resulting mass determination or mass spectrum may provide information which may be used to indicate a disease state, or propensity to disease, uniquely identify the source of the sample, or map locations in a genome.

In yet another embodiment, methods are provided for detecting polymorphisms in at least one amplified target nucleic acid further comprising removing at least one flanking polynucleotide region, if present, from at least one of the amplified target nucleic acids before the isolating step.

In a further embodiment, methods for detecting polymorphisms are described wherein the isolating step comprises binding the amplified target nucleic acid to a solid support and the removing step comprises using one or more restriction endonucleases to cleave off one or more flanking polynucleotide regions.

The mass of a preferably single-stranded amplified target nucleic acid may be compared with the known or predicted mass of the corresponding wild type single-stranded amplified target nucleic acid, that is, the wild type version of the target nucleic acid that is being screened for polymorphism. Alternatively, the masses of more than one amplified target nucleic acid can be compared with the known or predicted masses of the corresponding wild type amplified target nucleic acids.

The amplified target nucleic acid or set thereof, can optionally have one or more nucleotides replaced with mass-modified nucleotides, including mass-modified nucleotide analogs. For example, FIG. 2A and FIG. 2B illustrate the increase in resolution for a A to T mutation where the mass-modified nucleotide heptynyldeoxyuridine has been used in place of T during PCR amplification. The use of this mass-modified nucleotide results in a separation of mass spectral peaks of 65 mass units instead of only 9 mass units. As this example illustrates, mass-modified nucleotides of the present invention may effect substantial increases in spectral resolution with only relatively small modifications in mass. Other examples of mass-modified nucleotides useful in the present invention include 5-(3-aminoallyl)-2'-dUTP, 5-bromo-dCTP, 5-iodo-dCTP, 7-methyl-dGTP, 7-deaza-dGTP, dITP, 5-bromo-dUTP, 1,N6-etheno-dATP, 5-mercuri-dCTP, aminomethylcoumarin-6-dUTP, biotin-16-dUTP, 5-methyl-dCTP, 7-deaza-dATP, alphathio-dNTPs, n6-aminohexyl-dATP, 5-iodo-dUTP.

Another optional aspect of the invention is the inclusion of internal calibrants or internal self-calibrants in the amplified target nucleic acid or set thereof to be analyzed by mass spectrometry to provide improved mass accuracy.

A preferred aspect of the invention includes the methods of detecting polymorphisms where the determining step further includes utilizing internal self-calibrants to provide improved mass accuracy. The isolation methods separately or together may also be combined with the use of internal self-calibrants.

The above methods, separately or in combination, may also be combined with the use of mass-modified nucleotides and mass-modified nucleotide analogs incorporated in the single-stranded or double-stranded amplified target nucleic acid or set of single-stranded or double-stranded amplified target nucleic acids to improve mass resolution between mass peaks. The methods of detecting polymorphisms may also include at least one single-stranded amplified target nucleic acid optionally having one or more nucleotides replaced with mass-modified nucleotides.

In another embodiment, kits for preparing amplified target nucleic acids for mass spectrometric analysis are provided. The kits of the invention may include a first primer capable of binding a first strand of one of the target nucleic acids at a region 5' to a region of interest of said target nucleic acid; a second primer capable of binding a second strand complementary to the first strand at a region 5' to the region of interest of the target nucleic acid; a DNA polymerase capable of extending the primers to form primer extension products of the first and second primers; and a restriction endonuclease capable of reducing the length of amplified target nucleic acids where the first and second primers and said DNA polymerase are provided in a concentration and buffer suitable for increasing the number of target nucleic acids to form amplified target nucleic acids

Another embodiment encompasses a kit for preparing a double-stranded target nucleic acid having a first strand and a second complementary strand for mass spectrometric analysis including: a first primer capable of binding the first strand of the target nucleic acid 5' to a region of interest of the target nucleic acid; a second primer capable of binding the second strand of the target nucleic acid 5' to the region of interest of the target nucleic acid; a DNA polymerase capable of extending the primers to form an amplified target nucleic acid; and a restriction endonuclease capable of reducing the length of the amplified target nucleic acid.

The first and second primers and DNA polymerase may be provided in a concentration and buffer suitable for increasing the number of target nucleic acids to form amplified target nucleic acids. The restriction endonucleases may be Type II or Type IIS restriction endonucleases. Preferably, the first primer is biotinylated, preferably at or near the 5' end and the kit further comprises a solid support capable of selectively binding the first strand of the amplified target nucleic acid. Thus, where the first primer is biotinylated, the solid support could be a streptavidin bead. Kits included in this invention may preferably also comprise a matrix, such as 3-hydroxypicolinic acid.

An aspect of the present invention also includes a kit for preparing a double-stranded target nucleic acid having a first strand and a second complementary strand for mass spectrometric analysis comprising: a first primer capable of binding the first strand of the target nucleic acid 5' to a region of interest of the target nucleic acid; a second primer capable of binding the second strand 5' to the region of interest of the target nucleic acid; and a DNA polymerase capable of extending the primers to form an amplified target nucleic acid, where the first primer comprises a cleavable primer cleavable by chemical or enzymatic treatment. Preferred cleavable primers include those having an exonuclease blocking moiety, a Type II or Type II restriction endonuclease recognition site, or a chemically cleavable site, such as a modified base, a modified sugar, or a chemically cleavable group incorporated into the phosphate backbone. Preferred chemically cleavable groups are dialkoxysilane, 3'-(S)-phosphorothioate, 5'-(S)-phosphorothioate, 3'-(N)-phosphoroamidate, or 5'-(N)-phosphoroamidate.

Preferably, the kit may also contain a solid support capable of selectively binding the first strand of the amplified target nucleic acid. For example, if the first strand preferably comprises a biotin, the solid support could comprise a streptavidin bead. These kits may also preferably further comprise a matrix, such as 3-hydroxypicolinic acid.

Another embodiment is a kit containing: a first primer capable of binding a first strand of one of the target nucleic acids at a region 5' to a region of interest of the target nucleic acid; a second primer capable of binding a second strand complementary to the first strand at a region 5' to the region of interest of the target nucleic acid; a DNA polymerase capable of extending the primers to form primer extension products of the first and second primers, where at least one of the two primers is a cleavable primer.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a resolved spectrum of nucleic acid fragments (DNA) in the 20,000 to 25,000 Da range using MALDI-TOF mass spectrometry. This positive ion time of flight mass spectrum was obtained from 200 fmoles of DNA in 3-HPA the summation of 100 laser pulses at 266 nm. The spectrum is of a single-stranded 72-mer which also shows a 71-mer. The FWHM resolution is 240 clearly resolving matrix adducts (labeled M).

FIG. 1B displays the positive ion TOF mass spectrum of a 88-mer parent peak and has a resolution of 330. This MALDI-TOF spectrum is a sum of 100 laser pulses at 266 nm was obtained from 200 fmoles of DNA in 3-HPA.

FIG. 2A shows the mass spectrum of a heterozygous mix of wild type and mutant DNA fragments where an A has mutated to a T giving spectral peaks separated by 9 mass units.

FIG. 2B illustrates the effect on mass resolution of a mass-substituted base. The spectrum in FIG. 2B consists of a mass spectrum of a heterozygous mix of wild type and mutant DNA fragments where A has mutated to T and the T has been replaced by heptynyldeoxyuridine during amplification of the mutant region (R = heptynye). The spectral peaks are now separated by 65 mass units as compared to only 9 mass units in FIG. 2A.

FIG. 3 is a diagram illustrating the effect of analyzing full-length double-stranded amplified target nucleic acid, where the blunt-ended double-strands result in unresolved peaks in the mass spectrum of the unresolved double-stranded fragments. In this instance, the source nucleic acid may be amplified, for example, by PCR, and then mass analyzed as the full-length, double-stranded product. The amplified target nucleic acid should typically be no greater than about 100 base pairs in length.

FIG. 4 is a diagram illustrating the effect of analyzing reduced-length double-stranded amplified target nucleic acid, where one of the strands has a 4 nucleotide overhang which results in fully resolved peaks of the double-stranded fragments in the mass spectrum. In this case, the source nucleic acid is amplified (*e.g.* PCR) in step (a), then reduced in length by restriction digestion of the double-stranded product in step (b) to yield an uneven ended product. The reduced-length, double-stranded product is then mass analyzed to yield to fully resolved peaks.

FIG. 5 illustrates that analyzing only a single-stranded amplified target nucleic acid reduces the number of strands and simplifies the mass spectrum. The source nucleic acid is amplified (*e.g.* PCR) in step (a) and then captured to a solid phase streptavidin bead in step (b). The solid phase is then rigorously washed to remove salts and unwanted biomolecules including

the bottom complementary strand in step (c). The isolated full-length, single-stranded amplified target strand is finally released into solution to be mass analyzed in step (d). Although this diagram as well as the diagrams in FIG. 6, FIG. 7, FIG. 8, FIG. 9, FIG. 10, FIG. 11 and FIG. 12 depicts the use of a biotinylate top primer (B) and a streptavidin solid support, these schemes are also generally applicable to other methods of selectively binding one strand of an amplified target nucleic acid to a solid support to facilitate the washing away of unwanted biomolecules, salts and the other complementary strains.

FIG. 6 employs a cleavable primer to reduce in length an amplified target nucleic acid that is larger than 100 base pairs to less than 100 nucleotides in length. The cleaving at the cleavable primer site also releases the single-stranded amplified target nucleic acid from the solid support. In this instance, the top primer is biotinylated and cleavable. In step (a) the source nucleic acid is amplified (*e.g.* PCR) and captured to solid phase streptavidin bead in step (b). The solid phase is then rigorously washed to remove salts and unwanted biomolecules including the bottom complementary strand in step (c). The isolated, reduced-length, single-stranded amplified target is released into solution by cleaving the primer at the cleavable site in step (d) for mass analysis.

FIG. 7 is a diagram illustrating the isolation of a single-stranded amplified target nucleic acid that has been reduced in length by cleaving off at least a portion of both flanking regions. The first flanking region contains a cleavable site in the cleavable primer, located outside of the region of interest. The second flanking region is on the opposite end of the amplified target nucleic acid and the portion of that second flanking region is cleaved off by digestion with a restriction endonuclease. The top primer is biotinylated and cleavable. The source nucleic acid is first amplified (*e.g.* PCR) (step (a)) and captured to the solid phase (*e.g.* streptavidin bead) (step (b)). The double-stranded target is then selectively restricted outside the genetic region of interest (step (c)). The order of steps (b) and (c) may be reversed. The solid phase is rigorously washed to remove salts and unwanted biomolecules including the bottom complementary strand (step (d)). Finally, the isolated, reduced-length, single-stranded amplified target strand is released into solution to be mass analyzed by cleaving the cleavable primer (step (e)).

FIG. 8 shows the isolation of a single-stranded amplified target nucleic acid, where the length of the amplified target nucleic acid is reduced by cleaving off a portion of both flanking regions by (1) using a first or top primer having a chemically cleavable site incorporated during amplification; and (2) using a bottom primer having a Type IIS restriction endonuclease

recognition site at the 5' end during amplification. The target nucleic acid is first amplified (*e.g.*, PCR) (step (a)). The use of the bottom primer with a type IIS restriction enzyme recognition site on the 5' end allows for the incorporation of this site on the end of the target nucleic acid. The top primer is then captured to a solid phase (*e.g.*, a streptavidin bead) in step (b). One flanking region is then cleaved off by digesting with a Type IIS restriction endonuclease in step (c). After rigorously washing to remove salts and unwanted biomolecules, including the unwanted (unbound) complementary strand in step (d), the single-stranded amplified target nucleic acid is released from the solid support by cleaving at the cleavable site within the cleavable primer in step (e) for mass spectral analysis. The order of steps (b) and (c) are reversible.

FIG. 9 depicts another embodiment of the invention, wherein one primer has an exonuclease blocker (•). After amplification of the target nucleic acid, step (a), the amplified target nucleic acid contains an exonuclease blocking group. The amplified target nucleic acid is then treated with a 5' to 3' exonuclease, step (b), which degrades the strand containing the exonuclease blocking group only up to the blocking group. The 5' to 3' exonuclease completely degrades the other complementary strand of the amplified target nucleic acid as the other strand does not have an exonuclease blocking group. The treatment with the 5' to 3' exonuclease, thus, leaves a single stranded amplified target nucleic acid for mass spectrometric analysis.

FIG. 10 is a diagram illustrating yet another embodiment, in which one primer contains a Type IIS restriction recognition site and a binding moiety, *e.g.*, biotin (B), wherein the Type IIS restriction cleavage site is located between the Type IIS restriction recognition site and the binding moiety. The source nucleic acid is first amplified using this primer and another primer complementary to the other strand, step (a). The amplified target nucleic acid is then restricted with the Type IIS restriction endonuclease corresponding to the Type IIS restriction recognition and cleavable sites in the primer (step (b)), leaving a reduced-length amplified target nucleic acid comprising a binding moiety, *e.g.* biotin, which can then be captured to a solid phase (streptavidin bead) (step (c)). The reduced-length amplified target nucleic acid is then rigorously washed to remove salts and the unbound complementary strand, step (d). Then the reduced-length, single-stranded amplified target nucleic acid is released from the solid support for mass spectrometric analysis by denaturing the biotin streptavidin bond, *e.g.*, by boiling under low salt conditions, step (e).

FIG. 11 is a diagram illustrating a variation of the embodiment illustrated in FIG. 10, wherein instead of isolating the bound reduced length, single-stranded amplified target nucleic

acid, the complementary (unbound) strand is released from the bound strand and isolated for mass spectrometric analysis. Thus, the source nucleic acid is amplified (step (a)). The amplified target nucleic acid is then cleaved with a type IIS restriction endonuclease (step (b)) and captured to a solid phase (*e.g.*, by biotin (b) interacting with a streptavidin bead) (step (c)). Unwanted salts and biomolecules are removed by washing (step (d)). This time, in contrast to the schemes depiction FIG. 5, FIG. 6, FIG. 7, FIG. 8 and FIG. 10. The initial washing is not so rigorous as to disrupt the interaction between the bound strand and its complement. In step (e) the complementary strand is released for mass analysis.

FIG. 12 is another embodiment wherein the double stranded DNA is mass analyzed. The source nucleic acid is amplified (step (a)). In this embodiment the top primer is biotinylated and contains a type IIS restriction site such that the cleavage site is between the region of interest and the biotin moiety. The amplified target nucleic acid is captured to the solid phase (step (b)) to facilitate washing away unwanted salts and biomolecules (step (c)). A restriction endonuclease is then used to release the double stranded fragment for mass spectral analysis (step (d)).

FIG. 13 is a mass spectrum of single-stranded amplified short tandem repeats from the tyrosine hydroxylase gene TH01 locus.

FIG. 14 is a mass spectrum of an allelic set (ladder) of single-stranded amplified target nucleic acids from the TH01 gene locus, wherein the single-stranded amplified target nucleic acids ranged in length from 71 to 95 nucleotides in length. The method used to produce this spectrum is the one depicted in FIG. 6.

FIG. 15 is a mass spectrum of a set of single-stranded amplified target nucleic acids wherein the single-stranded amplified target nucleic acids were the same as those depicted in FIG. 14 except that the lengths of the amplified target nucleic acids had been reduced by 31 base pairs by endonuclease cleavage. The method employed to prduce this spectrum is the one illustrated in FIG. 7.

FIG. 16A shows the chemical formula for 2'-deoxythymidine-3'-(S)-phosphorothioate

FIG. 16B shows the chemical formula for 2'-deoxythymidine-5'-(S)-phosphorothioate.

DESCRIPTION OF SPECIFIC EMBODIMENTS.

The present invention, directed to methods of and fkits for preparing target nucleic acids for mass spectrometric analysis and for detecting polymorphisms. provides advantages of technical ease, speed, and high sensitivity. Additionally, only minute samples of femtomole

amounts are required. The methods and kits described herein yield a minimal set of products with improved mass resolution and accuracy and detailed information about the nature of the polymorphisms detected in the target nucleic acids screened.

One embodiment of the present invention involves methods of detecting polymorphisms in one or more target nucleic acids comprising (a) amplifying at least one of said target nucleic acids, wherein each of said target nucleic acids comprises a region of interest and optionally one or more flanking regions, (b) isolating either a positive or a negative strand of interest of each of said target nucleic acids in the form of one or more single-stranded amplified target nucleic acids, wherein said isolating preferably comprises binding said strand of interest of each of said amplified target nucleic acids to a solid support, and (c) determining the masses of each of said single-stranded amplified target nucleic acids using a mass spectrometer, wherein said determining preferably does not involve sequencing of said amplified single-stranded target nucleic acids. The amplifying step may include the use of a specialized primer that can be used in the isolating step to bind the amplified target nucleic acids to a solid support. The primer may also have attached a cleavable or reversible linker, or the primer itself may contain a cleavable site. If a cleavable site is introduced into one of the amplified target nucleic acids by using a cleavable or reversible linker during said amplifying step, the determining does not involve sequencing of the amplified target nucleic acids. The primer may also be biotinylated or modified in other ways such as to effect binding of the amplified target nucleic acids to a solid support. The primer may also optionally be bound or attached to the solid support prior to being amplified. One of ordinary skill in the art will appreciate the multiplicity of methods to effect such attachment.

The isolating may further comprise denaturing and washing to remove the complementary strand from the strand of interest which is bound to a solid support, followed by release of the bound single-stranded target nucleic acids from the solid support. Alternatively, the unbound complementary strand may be released and isolated for mass spectrometric analysis.

After amplifying and either before or after the amplified target nucleic acids have been bound to a solid support, the amplified target nucleic acid may be reduced in length by a number of different techniques. For example, one or more flanking regions may be cleaved using one or more restriction endonucleases, such as Type II or Type IIS restriction endonucleases or combinations thereof. The amplified target nucleic acid may also be reduced in length by using a cleavable primer. Another method of reducing length comprises using an exonuclease blocking

moiety in one of the two primers for amplification, and digesting said amplified target nucleic acid with a 5' to 3' exonuclease.

The target nucleic acid may be single-stranded or double-stranded DNA, RNA or hybrids thereof, from any source. The target nucleic acid is generally a nucleic acid which must be screened to determine whether it contains a polymorphism. The corresponding target nucleic acid derived from a wild type source is referred to as a wild type target nucleic acid. The amplified target nucleic acids can be obtained from a source sample containing nucleic acids and can be produced from the nucleic acid by PCR™ amplification or other amplification techniques. Although human sources are preferred, any source which one is interested in screening for polymorphisms may be used in the methods described herein. When the target nucleic acid is RNA, the RNA strand is the + strand. If desired, the target nucleic acid may be an RNA/DNA hybrid, wherein either strand can be designated the + strand and the other, the - strand.

In cases where the amplified target nucleic acid contains RNA, the methods using restriction endonucleases described herein cannot be used to directly reduce the length of the final product. A restriction endonuclease may be used to reduce the length of the double-stranded DNA intermediates prior to the RNA transcription step.

The amplified target nucleic acids are typically less than 100 bases in length because current mass spectrometric methods do not have the mass accuracy and resolution necessary to identify a single base change in polynucleotides larger than 100 base pairs. However, as mass spectrometric techniques for analyzing nucleic acids improve, the single-stranded or double-stranded amplified target nucleic acids of this invention may be larger than 100 bases in length.

Due to the simpler mass spectrum that results from mass analysis of single-stranded amplified target nucleic acids, it is preferred to determine the masses of sets of single-stranded amplified target nucleic acids. The amplified target nucleic acids may also contain mass-modified nucleotides, which can enhance ease of analysis, especially when a point polymorphism has resulted in a very small mass change (on the order of 9 Da) in a target nucleic acid as compared to the corresponding wild type target nucleic acid. The methods described herein use mass spectrometry to determine the masses of a single-stranded amplified target nucleic acid or set of single-stranded amplified target nucleic acids to detect polymorphisms in at least one target nucleic acid.

The amplified target nucleic acids comprise a region of interest and optionally, one or more flanking regions. A region of interest contains or is suspected of containing a

polymorphism, whereas a flanking region is generally believed not to contain a polymorphism or a polymorphism in that region is considered unimportant. The region of interest may be as small as a single nucleotide. A flanking region may contain a cleavable site or cleavable moiety that can be selectively cleaved to release single-stranded nucleic acids from a solid support prior to mass spectrometric analysis. An amplified target nucleic acid may also optionally comprise another flanking region on the end of the target nucleic acid opposite from the cleavable site used for release from the solid support. This second flanking region may contain one or more restriction cleavable sites that do not occur in the region of interest.

The methods described herein may be performed on a single amplified target nucleic acid or on a set of different amplified target nucleic acids, each containing a different region of interest. The various steps of reducing length, binding to a solid support, releasing from the solid support may differ with respect to each different target nucleic acid in a set, or may be the same, and the resulting set of single-stranded or double-stranded amplified target nucleic acids can be mass analyzed simultaneously. Accordingly, another advantage of the methods described herein is that they can be used to prepare a set or collection of two or more different target nucleic acids in a single reaction or a single container, possibly using at least one common reagent, which results in increased efficiency and more informative data from a single mass spectrum of the prepared target nucleic acids.

Wild type refers to a standard or reference nucleotide sequence, or number of repeat di-, tri-, or tetra-nucleotides, to which variations are compared. As defined, any variation from wild type is considered a polymorphism, including naturally occurring sequence polymorphisms, and mutations which are pathogenic.

Two nucleic acids are considered "complementary" if they are capable of specifically hybridizing to one another (i) under typical hybridization and wash conditions (see, *eg.*, Maniatis *et al.*, 1982) or (ii) using reduced stringency wash conditions that allow at most about 25-30% base pair mismatches, for example, 2 x SSC, 0.1% SDS, room temperature twice, 30 minutes each; then 2 x SSC, 0.1% SDS, 37°C once, 30 minutes; then 2 x SSC room temperature twice, 10 minutes each.

The types of mass spectrometry used in the invention include ESI or MALDI, wherein these methods may optionally include time-of-flight. The significant multiple charging of molecules in ESI and the fact that complex mixture analysis is often required mean that the ESI mass spectra will consist of a great many spectral peaks, possibly overlapping and causing

confusion. Because the MALDI MS approach produces mass spectra with fewer major peaks, this method is preferred. Thus, a MALDI MS time-of-flight instrument is preferred for the mass analysis of the invention.

The methods described herein do not require sequencing of one or more target nucleic acids (using the sequencing methods that require four different base-specific chain termination reactions or chemical cleavages to determine the complete nucleotide sequence of a nucleic acid) in order to determine the nature and presence of a polymorphism within any of said target nucleic acids. However, the methods described herein, separately or in combination, may be used in the sequencing of one or more amplified target nucleic acids using mass spectrometric techniques.

For an initial polymorphism screen, a useful range of amplified target nucleic acid sizes that will allow detection of a point polymorphism is around 10 to 100 bases. This size range is where mass spectrometry presently has the necessary level of mass resolution and accuracy. Thus, the methods used in this invention are designed to produce amplified target nucleic acids ranging up to about 100 bases in size, but can also be used to produce larger amplified target nucleic acids.

Existing mass spectrometric instrumentation in the case of MALDI-TOF MS optimally has a mass accuracy of about 1 part in 10,000 (0.01%), four times what is necessary for detecting a single base change in a 50-base long single-stranded DNA fragment. Utilization of mass-modified nucleotides (described herein) and nearby masses as internal calibrants, provides optimal resolution and mass accuracy of larger nucleic acids, and can extend the usable point polymorphism detection range up to 100 bases, if not higher. Continued advances in mass spectrometric instrumentation will also push this range higher.

Examples of the resolving capabilities of MALDI-TOF MS are displayed in FIG. 1A and FIG. 1B which show the positive ion TOF mass spectra obtained from 200 fmoles of DNA in the matrix 3-HPA. FIG. 1A shows two single-stranded PCR products of lengths 71 and 72 (mass difference = 305 Da = Adenosine) as well as the 72mer and 72mer + a single matrix adduct (M) (mass difference = 139 Da) to be well resolved (FWHM resolution = 240). FIG. 1B shows an 88 base length single-stranded product having a resolution of 330. Both spectra display high enough accuracy and resolution to detect a point polymorphism if one were present.

BENEFITS OF ANALYZING SINGLE-STRANDED NUCLEIC ACIDS

One object of this invention is the accurate mass determination of a single-stranded amplified target nucleic acid or a set of single-stranded amplified target nucleic acids to determine presence and character of any polymorphisms. The embodiments of this invention include mass spectrometric mass determination of the single-stranded amplified target nucleic acid or set of different single-stranded amplified target nucleic acids, as well as mass determination of a mass-modified, single-stranded amplified target nucleic acid or set thereof. A preferred embodiment is to detect polymorphisms in an amplified target nucleic acid in single-stranded form, wherein the single-stranded amplified target nucleic acid(s) are derived from one of either the positive or the negative strand of the genome. The examples of single-stranded methods described herein focus on single-stranded amplified target nucleic acids derived from the positive strand, although the methods disclosed in the present invention are equally applicable to target nucleic acid as derived from the negative strand as well.

FIG. 3 illustrates that a double-stranded target nucleic acid comprising two complementary strands, produces two difficult to resolve peaks in the mass spectrum corresponding to the denatured single strands. The additional peaks from double-stranded amplified target nucleic acids as compared to single-stranded amplified target nucleic acids add to the congestion of mass peaks in the mass spectra, as well as introducing the possibility that it may be extremely difficult to resolve the complementary fragments if they have nearly or exactly identical base compositions. Furthermore, some portion of the double-stranded amplified target nucleic acids do not fully denature, and mass peaks corresponding to the double-stranded products increase the spectral congestion.

Spectra using both strands may also contain a two-fold redundancy in data, since any polymorphism in one strand may be present within its complement. One strand my be removed prior to mass spectrometric analysis while still producing all data necessary for a complete polymorphism analysis. Therefore, it is often preferable to analyze a set of single strands, using only one of the two complementary sets of amplified target nucleic acids representing the full set of target nucleic acids.

FIG. 4 shows the expected spectrum if only the positive strand of a target nucleic acid from FIG. 3 is analyzed by mass spectrometry as analysis of one of the two complementary strands of the double-stranded amplified target nucleic acids halves the number of expected peaks within the mass spectra, allowing for better resolution. Thus, removal of one of the two

strands from each amplified target nucleic acids eliminates the greatest source of complication for each spectra. A number of methods for isolating and preparing single-stranded amplified target nucleic acids for mass spectrometry are described herein.

PURIFICATION METHODS

For mass spectrometry analyses, the target nucleic acids should be within the resolvable range and high mass accuracy range of the mass spectrometer. Additionally, nucleic acid fragments that do not contribute to the analysis and may unnecessarily convolute the mass spectra should be eliminated, if feasible.

With analysis methods such as gel electrophoresis, a mixture of specifically labeled nucleic acid fragments (radioactively or fluorescently tagged) can be visualized in the presence of other unlabeled nucleic acid fragments that comigrate but are invisible and therefore do not convolute analysis of the gel data. The mass spectrometric methods described herein do not use any form of labeling that could render certain fragments invisible, *e.g.* the complementary strand in a double-stranded product, and it is therefore preferable to remove such fragments prior to analysis.

The samples should preferably be of relatively high purity prior to introduction to the mass spectrometer. The presence of impurities, especially salts, may greatly affect the resolution, accuracy and intensity of the mass spectrometric signal. Contaminating primers, residual sample genomic DNA, and proteins, all can affect the quality of the mass spectra.

The purification methods of the present invention are well-suited to mass spectrometric analysis of nucleic acids. For example, the methods herein physically isolate selected sets of single-stranded or double-stranded amplified target nucleic acids from a multiplicity of impurities including undesirable nucleic acid fragments (including the complementary strand and flanking regions), proteins and salts, that would result in a poor quality mass spectrum. These isolation methods offer significant advantages due to the physical separation of a desired set of single-stranded or double-stranded amplified target nucleic acids from other impurities in preparation.

APPROACHES TO ISOLATING SINGLE-STRANDED OR DOUBLE-STRANDED AMPLIFIED TARGET NUCLEIC ACIDS

As described earlier, analysis of single-stranded amplified target nucleic acids is generally preferable since it provides a complete set of data with the minimal number of fragments and therefore simplifies the spectra and facilitates an increase in the total number of target nucleic acids that can be analyzed in a single assay. A number of approaches can be taken toward the production of single-stranded amplified target nucleic acids and their purification which includes the elimination of undesired oligonucleotides. In some cases, it may be preferable to use a method of amplification that yields primarily single-stranded amplified target nucleic acids, such as asymmetric PCR or transcription-mediated amplification.

To isolate the single-stranded amplified target nucleic acids, the amplified target nucleic acids may be designed to be attached or bound to a solid support. Several means are available to effect this attachment to a solid support, including: (a) hybridization to a complementary, solid-phase bound nucleic acid capture probe (which can be an oligonucleotide or one strand of the amplified target nucleic acid) comprising a first binding moiety that specifically binds to a second binding moiety attached to a solid phase; (b) direct binding of the amplified target nucleic acid strands of interest, each comprising a polynucleotide region of interest and a first binding moiety, to a second binding moiety attached to a solid phase (*e.g.* biotin/streptavidin or avidin or antigen/antibody pairs); or (c) direct covalent attachment of the strands of interest to a solid support.

A capture probe is an oligonucleotide that comprises a portion capable of hybridizing to a nucleic acid, such as an amplified target nucleic acid, and a binding moiety that binds the capture probe to a solid phase, either through covalent binding or affinity binding, or a mixture thereof. A capture probe can itself bind to a solid support via binding moieties (direct capture) or can bind to a solid support via another capture probe that binds to a solid support (indirect capture).

A preferred embodiment is the use of a biotinylated amplified target nucleic acid coupled to streptavidin or avidin attached to a solid support where the strand of interest is itself bound. Biotin coupling to streptavidin (or avidin) requires that any amplified target nucleic acid or acids contain a biotin. The biotin is part of the linker molecule. It is straightforward to capture the amplified target nucleic acid because biotinylated primers can be used in the PCR amplification. If only one of the two strands of an amplified target nucleic acid is to be analyzed by mass spectrometry, only one of the two PCR primers for each different target nucleic acid should be

biotinylated. For each target nucleic acid, the PCR primer to be biotinylated should be the primer that is extended to form the single-stranded amplified target nucleic acid of interest.

The amplified target nucleic acid or set of amplified target nucleic acids can be covalently attached to a solid support using any of the number of methods commonly employed in the art to immobilize an oligonucleotide or polynucleotide on a solid support. The amplified target nucleic acid or set of amplified target nucleic acids covalently attached to the solid support should also be stable and accessible for base hybridization.

Covalent attachment of the amplified target nucleic acid or set of amplified target nucleic acids to the solid support may occur by reaction between a reactive site or a binding moiety on the solid support and a reactive site or another binding moiety attached to the target or via intervening linkers or spacer molecules, where the two binding moieties can react to form a covalent bond. Coupling of an amplified target nucleic acid or set of amplified target nucleic acids to a solid support may be carried out through a variety of covalent attachment functional groups. Any suitable functional group may be used to attach the amplified target nucleic acid or set of amplified target nucleic acids to the solid support, including disulfide, carbamate, hydrazone, ester, N-functionalized thiourea, functionalized maleimide, mercuric-sulfide, gold-sulfide, amide, thiolester, azo, ether and amino.

The solid support may be made from a wide variety of materials, such as cellulose, nitrocellulose, nylon membranes, controlled-pore glass beads, acrylamide gels, polystyrene, activated dextran, agarose, polyethylene, functionalized plastics, glass, silicon, aluminum, steel, iron, copper, nickel and gold. Some solid support materials may require functionalization prior to attachment of an oligonucleotide or capture probe. Solid supports that may require such surface modification include aluminum, steel, iron, copper, nickel, gold, silicon, and nonfunctionalized polymers. Solid support materials for use in coupling to a capture probe include functionalized supports such as the 1,1'-carbonyldiimidazole activated supports available from Pierce (Rockford, IL) or functionalized supports such as those commercially available from Chiron Corp. (Emeryville, CA). Binding of an amplified target to a solid support can be carried out by reacting a free amino group of an amino-modified target with the reactive imidazole carbamate of the solid support. Displacement of the imidazole group results in formation of a stable N-alkyl carbamate linkage between the amplified target and the support.

The amplified target nucleic acid or set of amplified target nucleic acids may also be bound to a solid support comprising a gold surface. The amplified target nucleic acid or set of

amplified target nucleic acids can be modified at their 5'-end with a linker arm terminating in a thiol group, and the modified amplified target nucleic acid or set of modified amplified target nucleic acids can be chemisorbed with high affinity onto gold surfaces (Hegner, *et al.*, 1993b).

In methods in which a solid-phase approach is used, preferably the double-stranded amplified target nucleic acid or set of amplified target nucleic acids may be washed to remove deleterious contaminants. However, when the amplified target nucleic acid strands of interest are directly bound, either covalently or via biotin/streptavidin biotin/avidin interactions, it is preferable to rigorously wash the sample to yield the highest purity. Such a rigorous wash typically removes the complementary strand, if present, isolating the single-stranded amplified target nucleic acid. Following washing, it is necessary to release single-stranded amplified target nucleic acids from the solid support for mass spectrometric analysis. The isolation of a set of single-stranded amplified target nucleic acids may be performed on the same plate that is used within the mass spectrometer or on a separate surface such as beads or a filter. Both the capture probe hybridization and biotin/streptavidin or biotin/avidin approaches can use a number of means of denaturation to disrupt the noncovalent interactions and afford release of the set of single-stranded amplified target nucleic acids bound to the solid support.

Alternatively, a cleavable linkage may be incorporated between the first binding moiety and the amplified target nucleic acids. Any covalent coupling chemistry may be reversible or it may be necessary to include a separate chemically cleavable linkage somewhere within the bound product. It may also be useful to use a chemically cleavable linkage approach with the biotin/streptavidin (or avidin) strategies so that release of the double-stranded target nucleic acids can be performed under relatively mild conditions. In all cases the cleavable linkage can be located within the linker molecule connecting the biotin and the base (*e.g.* a disulfide bond in the linker), within the base itself (*e.g.* a more labile glycosidic linkage), or within the phosphate backbone linkage (*e.g.* replacement of phosphate with a phosphoramidate).

Another way to isolate single-stranded amplified target nucleic acids is to use a primer comprising an exonuclease blocking moiety and to treat with a 5' to 3' exonuclease, which digests the strand lacking an exonuclease blocking moiety and the portion of the other strand up to the exonuclease blocking moiety, leaving just the portion of the strand containing the exonuclease blocking moiety and the portion of that strand 3' to the exonuclease blocking moiety. This method is described in the methods of reducing length section herein. Single-

stranded amplified target nucleic acids can also be isolated by using a DNA-specific or RNA-specific nuclease to digest an RNA/DNA hybrid.

The use of two primers each comprising an exonuclease blocking moiety, wherein each primer binds to a different complementary strand, is another way to isolate a double-stranded amplified target nucleic acid.

METHODS OF REDUCING LENGTH OF AMPLIFIED TARGET NUCLEIC ACIDS

After the amplification of target nucleic acids, the amplified target nucleic acids, which are in double-stranded form, can be cleaved with restriction endonucleases to remove flanking regions that are not within the region of interest, wherein said region of interest is suspected of containing a polymorphism.

If DNA restriction endonucleases are used to remove one or more flanking regions from an amplified target nucleic acid prior to isolating the single-stranded or double-stranded amplified target nucleic acid(s), it may be necessary for the amplified target nucleic acid to have a double-stranded form prior to restriction, or more specifically, that the restriction endonuclease recognition sites and cleaving sites be located in double-stranded DNA regions flanking or outside the region of interest. The alternative to having fully double-stranded DNA prior to restriction is to hybridize restriction site oligonucleotide probes to single-stranded DNA, wherein the restriction site oligonucleotide probes are complementary to the restriction sites for selected restriction endonucleases.

The basic known methods for DNA isolation - precipitation, dialysis, filtration and chromatography do not isolate single-stranded from double-stranded DNA. If these purification methods are employed, and it may be desired to produce a single-stranded product, it is necessary to add a separate step where single-strand isolation is performed.

If restriction endonucleases are used to cleave off one or more regions from an amplified target nucleic acid, a preferred method for isolating single-stranded amplified target nucleic acids from these products is to use at least one biotinylated primer located at one end of an amplified target nucleic acid.

The production of reduced length amplified target nucleic acids can provide benefits of increased accuracy and resolution in the mass spectrometric analysis of even double-stranded amplified target nucleic acids. Double-stranded amplified target nucleic acids can have their length reduced in a similar manner to that used for processing single-stranded amplified target

nucleic acids. Either endogenous restriction recognition sites outside the region of interest or primer-incorporated restriction recognition sites, as described below, or combinations thereof can be used. Endogenous restriction recognition sites are those that are found naturally within one or more flanking regions.

In cases where one or more endogenous restriction recognition sites cannot be found outside the region of interest, an alternative method is necessary for reducing the length of the amplified target nucleic acid. Use of a modified primer during the amplification process can mediate the incorporation of a Type II or Type IIS restriction endonuclease recognition site within a primer region of the amplified target nucleic acid. Type IIS restriction endonucleases recognize a particular double-stranded sequence region and selectively cleave the double strand a defined distance away from the recognition site. As an example, the restriction enzymes *BpmI* and *BsgI* cleave the double strands 14 nucleotides (top strand) and 16 nucleotides (bottom strand) away from the recognition sites. Other representative Type IIS restriction enzymes include *BseRI, BsmRI* and *FokI*. See New England Biolabs 1996 Product Catalog. Use of Type IIS restriction for the reduction of amplified target nucleic acids is illustrated in FIG. 7.

The restriction method for reducing the length of an amplified target nucleic acid affords significant advantages where double-stranded amplified target nucleic acids are to be analyzed by mass spectrometry. For instance, the smaller molecules are easier to resolve. Moreover, a second beneficial effect of using restriction endonucleases to reduce length, specifically one that does not produce blunt ends, is the production of two strands of different lengths and hence different masses. The creation of two complementary strands of different lengths, *e.g.* 4 to 6 nucleotides difference in size, yields dramatically improved separation and resolution of two complementary strands during mass spectrometric analysis as shown in FIG. 4. In many cases, reduction of length by restriction endonuclease digestion can eliminate the need for single-strand isolation.

In one embodiment, the restriction endonuclease recognition site can be the same as the site of cleavage, located in the flanking region opposite from the end of the amplified target nucleic acid that is bound to the solid support. In another embodiment, the restriction endonuclease recognition site is different from the site of cleavage, as in the case of Type IIS restriction endonucleases, which cleave at a defined distance (20-40 bases) from one side of their recognition sequence. When a Type IIS restriction endonuclease is used to reduce the length of

an amplified target nucleic acid, both the recognition site and the site of cleavage are commonly located outside of the region of interest and in the flanking region.

One of ordinary skill will readily appreciate that many combinations and variations of these methods for reducing length are possible. For example, endogenous Type II restriction recognition sites for one or more Type II restriction endonuclease can be used to reduce length on one or both regions flanking a region of interest. Alternatively, endogenous Type II and endogenous Type IIS restriction recognition and cleavage sites can be used to reduce length by cleaving in one or more flanking regions. Also, one or more restriction recognition sites can be introduced using a mismatch primer or an overhang primer containing one or more new restriction recognition sites. A mismatch primer is one which contains at least a single base mismatch with the target nucleic acid to be amplified and can include primers that have an overhang region that does not hybridize to the target nucleic acid. Mismatch primers are a type of cleavable primer. Alternatively, endogenous restriction recognition sites and primer-introduced restriction recognition sites can be combined. Cleavable sites can also occur outside the primer region, ranging from 20-50 nucleotides away from the end of the primer region. All of these types of primers are cleavable primers because they contain a site, moiety or group, that can be used to reduce the length of the target nucleic acid. For example, cleavable primers may contain a recognition site, a cleavable site, or an exonuclease blocking moiety.

Another method of reducing length involves the use of a primer comprising an exonuclease blocking moiety, wherein said exonuclease blocking moiety prevents a 5' to 3' exonuclease from digesting a region of interest that is 3' to said exonuclease blocking moiety. The exonuclease blocking moiety can include modified nucleotides that prevent 5' to 3' exonuclease activity from continuing, *e.g.* phosphorothioates, methyl phosphonates, borano phosphates, and peptide nucleic acids (PNA). FIG. 9 depicts the use of an exonuclease blocking moiety and an exonuclease to reduce the length of a target nucleic acid. The nucleotides that are degraded by the exonuclease are a multiplicity of cleavable sites, including many that are adjacent to one another.

Peptide nucleic acids are modified DNA mimics in which the sugar-phosphate backbone has been replaced with a backbone based on amino acids. Peptide nucleic acids exhibit sequence-specific binding to DNA and RNA and are resistant to nuclease and protease attack. See Buchardt *et al.*, 1993. A preferred 5' to 3' exonuclease is Exonuclease III. FIG. 9 illustrates

how the use of this method results in isolation of a reduced-length single-stranded amplified target nucleic acid.

The exonuclease approach to reducing length can be also used in combination with one or more of the restriction endonuclease cleavage techniques described above to reduce length. In such a combined approach, the restriction cleavage should occur before the exonuclease digestion.

One of ordinary skill in the art will appreciate that the above methods of reducing length may also be used as means of isolating a reduced-length amplified target nucleic acid or a reduced-length single-stranded amplified target nucleic acid. For example, a chemically cleavable site can be incorporated in a flanking region and cleavage at that site can accomplish both length reduction and release from a solid support at the same time. Thus, for example, after the amplified target nucleic acid is reduced in length, either the bound strand or the unbound strand or both strands of said amplified target nucleic acid can be isolated for mass spectrometric analysis.

In cases where the single-stranded amplified target nucleic acid strand to be analyzed is directly bound to the solid phase, it can be rigorously washed to remove unbound components, including any number of deleterious contaminants, including the unwanted complementary strand, nucleic acid fragments containing at least a portion of a flanking region, salts, enzymes, and other reagents. These unbound components can be removed from any nucleic acid bound to a solid support in any of the embodiments described herein and combinations thereof. If the strand to be analyzed (the strand of interest) is not bound directly but rather via hybridization to a complementary nucleic acid, it cannot be as rigorously washed and thereby cannot be purified to as great an extent. Direct binding of the amplified target nucleic acid strand to be analyzed ultimately produces a higher quality signal, *e.g.* less salt adducts, during mass spectrometric analysis, thus improving mass resolution and accuracy.

Following the necessary wash steps, the single-stranded amplified target nucleic acids are released from the solid support and analyzed by mass spectrometry. Note that regions that are cleaved off by one or more restriction endonucleases are released into solution and washed away, and are therefore not analyzed. Loss of these flanking regions can enhance the ability for mass spectrometry to quickly identify the existence of a polymorphism. The isolation of the single-stranded amplified target nucleic acids occurs prior to the mixing of the single-stranded amplified target nucleic acids with the matrix material for mass spectrometric analysis.

To release the reduced-length single-stranded amplified target nucleic acid from the solid support, several methods can be used, depending on the methods used to bind the amplified target nucleic acid to the solid support. Both the hybridization and biotin/streptavidin (or avidin) methods can use a number of means of denaturation to disrupt noncovalent interactions and cause the release of the bound single-stranded amplified target nucleic acids. Any covalent chemistry used must be either reversible or include a separate chemically cleavable site somewhere within the bound product. It may be preferred to use a chemically cleavable site with the biotin/streptavidin (or avidin) method so that release of the target nucleic acids can be performed under relatively mild conditions. In all cases, the cleavable site can be located within a linker molecule connecting the biotin and the base (*e.g.* a disulfide bond in the linker), within the base itself (*e.g.* a more labile glycosidic linkage), or within the phosphate backbone linkage (*e.g.* replacement of phosphate with a phosphoramidate).

In a preferred embodiment, the cleavable site is located near the 3' end of the primer used to bind the amplified target nucleic acid to the solid support. By locating the cleavable site near the 3' end, it is possible to further reduce the length of the amplified target nucleic acid, eliminating a flanking region from the polynucleotide region of interest. Cleavable primers are described in PCT/US96/06116, filed April 26, 1996 (incorporated herein by reference).

IMPROVING MASS ACCURACY BY INTERNAL CALIBRATION AND INTERNAL SELF-CALIBRATION

Mass spectrometers are typically calibrated using analyses of known mass. A mass spectrometer can then analyze an analyte of unknown mass with an associated mass accuracy and precision. However, the calibration, and associated mass accuracy and precision, for a given mass spectrometry system (including MALDI-TOF MS) can be significantly improved if analyses of known mass are contained within the sample containing the analyte(s) of unknown mass(es). The inclusion of these known mass analyses within the sample is referred to as use of internal calibrants. External calibrants, i.e. analyses of known mass that are not mixed in with the set of target nucleic acids of unknown mass and simultaneously analyzed in a mass spectrometer, are analyzed separately. External calibrants can also be used to improve mass accuracy, but because they are not analyzed simultaneously with the set of target nucleic acids of unknown mass, they will not increase mass accuracy as much as internal calibrants do. Another disadvantage of using external calibrants is that it requires an extra sample to be analyzed by the mass spectrometer. For MALDI-TOF MS, generally only two calibrant molecules are needed for

complete calibration, although sometimes three or more calibrants are used. All of the embodiments of the invention described herein can be performed with the use of internal calibrants to provide improved mass accuracy.

Using the methods described herein, one can obtain a mass spectrum with numerous mass peaks corresponding to the set of single-stranded amplified target nucleic acids under study. If no polymorphism is present in any of said target nucleic acids, all of the mass peaks corresponding to the amplified target nucleic acids will be at mass-to-charge ratios associated with the set of amplified target nucleic acids from the wild type target nucleic acids. However, if a target nucleic acid contains a polymorphism, usually no more than one or two of the mass peaks will be shifted in mass, leaving the majority of mass peaks at unaltered locations. In a preferred embodiment of the invention, a self-calibration algorithm uses these nonpolymorphic or unmutated target nucleic acids for internal calibration to optimize the mass accuracy for analysis of the single-stranded amplified target nucleic acids containing a polymorphism, thus requiring no added calibrant(s), simplifying the calibration, and avoiding potential spectral overlaps. In a given sample, however, it will not be known *a priori* which mass peaks, if any, are altered or shifted from their expected masses for the wild type target nucleic acids.

The self-calibration algorithm begins by dividing up the observed mass peaks into subsets, each subset consisting of all but one or two of the observed mass peaks. Each data subset has a different one or two mass peaks deleted from consideration. For each subset, the algorithm divides the subset further into a first group of two or three masses which are then used to generate a new set of calibration constants, and a second group which will serve as an internal consistency check on those new constants. The internal consistency check begins by calculating the mass difference between the m/z values calculated for the second group of mass peaks and the values corresponding to reasonable choices for the associated wild-type target nucleic acids. The internal consistency check can thus take the form of a chi-square minimization where the key parameter is this mass difference. The algorithm finds which data subset has the lowest sum of the squares of these mass differences resulting in a choice of optimized calibration constants associated with group one of this data subset.

After new self-optimized calibration constants are obtained, the mass-to-charge ratios are determined for the mass peaks omitted from the data subset; these are the amplified target nucleic acids suspected to contain a polymorphism. The differences from the observed mass peaks for the wild type amplified target nucleic acids are then used to determine whether a

polymorphism is present, and if so, what the nature of this polymorphism is (*e.g.* the exact type of deletion, insertion, or point polymorphism). This self-calibration procedure should yield a mass accuracy of approximately 1 part in 10,000.

The methods described herein permit MALDI-TOF MS analysis of single-stranded amplified target nucleic acids which has a mass accuracy of approximately 1 part in 10,000. The use of internal self-calibrants makes it possible to extend this level of accuracy up to and potentially beyond 30,000 Da or 100 bases. This mass accuracy enables exact sizing of one or more target nucleic acids and the determination of the presence and nature of any polymorphism, including point polymorphisms, insertions and deletions. Further described herein are methods for improving the resolution of individual target nucleic acids by means including elimination of equal-length complementary pairs through the use single-strand-targeted isolation procedures, and the incorporation of mass-modified nucleotides to enhance the mass difference between similar sized amplified target nucleic acids and/or wild type amplified target nucleic acids. In addition, these methods provide for the removal of salts and other deleterious materials as well as a means for the removal of unwanted nucleic acid fragments prior to mass spectroscopic analysis.

Mass Resolution, Mass Accuracy, and the use of Mass-Modified Nucleotides

Any of the embodiments of the invention described herein optionally include amplified target nucleic acids having one or more nucleotides replaced with mass-modified nucleotides, wherein said mass-modified nucleotides comprise nucleotides or nucleotide analogs having modifications that change their mass relative to the nucleotides that they replace. The mass-modified nucleotides incorporated into the target nucleic acids of the invention must be amenable to the enzymatic and nonenzymatic processes used for the amplification of target nucleic acids. For example, the mass-modified nucleotides must be able to be incorporated by DNA or RNA polymerase during amplification of the target nucleic acid. Moreover, the mass-modified nucleotides must not inhibit the processes used to process the target nucleic acids, including, *inter alia*, specific cleavage by restriction endonucleases, whenever such steps are used. Mass-modifications can also be incorporated in the target nucleic acids of the invention after the enzymatic steps have been concluded. For example, a number of small chemicals can react to modify specific bases, such as kethoxal or formaldehyde.

Any or all of the nucleotides in the target nucleic acids can be mass-modified, if necessary, to increase the spread between their masses. It has been shown that modifications at the C5 position in pyrimidines or the N7 position in purines do not prevent their incorporation into growing nucleic acid chains by DNA or RNA polymerase (Lee *et al.*, 1992). For example, an octynyl moiety can be used in place of methyl on thymidine to alter the mass by 94 Da.

Mass-modifying groups can be, for example, halogen, alkyl, ester or polyester, ether or polyether, or of the general type XR, wherein X is a linking group and R is a mass-modifying group. The mass-modifying group can be used to introduce defined mass increments into the target nucleic acids. One of skill in the art will recognize that there are numerous possibilities for mass-modifications useful in modifying nucleic acid fragments or oligonucleotides, including those described in Oligonucleotides and Analogues: A Practical Approach, Eckstein ed. (Oxford 1991) and in PCT/US94/00193, which are both incorporated herein by reference.

At larger mass ranges (30,000-90,000 Da), the mass resolution and mass accuracy of current MALDI-TOF mass spectrometers will not typically be sufficient to identify a single base change. For this reason, it may be preferable to increase the useful mass range artificially by substituting standard nucleotides within a target nucleic acid with mass-modified nucleotides having significantly larger mass differentials. Use of mass-modified nucleotides applies as well to the mass range below 30,000 Da. Mass modification can generally increase the quality of the mass spectra by enlarging the mass differences between different amplified target nucleic acids of similar size and composition. For example, mass-modified nucleotides can increase the minimum mass difference between two amplified target nucleic acids that happen to be identical in base composition except for a single base which is an A in one and is a T in the other. Normally, these two target nucleic acids will differ in mass by only 9 Da. By replacing one of the bases with a mass-modified version during amplification, the mass difference can be > 20 Da.

The illustrations of spectra in FIG. 2A and FIG. 2B depict the influence mass-modified nucleotides can have on target nucleic acid resolution. One example of the many possible mass modifications useful in this invention is the use of 5-(2-heptynyl)-deoxyuridine in place of thymidine. The replacement of a methyl group by heptynyl changes the mass of this particular nucleotide by 65 Da. An A to T transversion in a nucleic acid in which all thymidine bases have been replaced with 5-(2-heptynyl)-deoxyuridine would produce a peak shift of 56 Da as opposed to 9 Da for the same nucleic acid fragments without the mass-modified nucleotides. The use of mass-modified nucleotides is especially important in the analysis of single-stranded target

nucleic acids derived from RNA. Normally, the masses of C and U vary by only 1 Da, making it practically impossible to detect C to U or U to C point polymorphisms within a given target nucleic acid.

Each of the techniques described herein can be used in combination with any of the isolation methods also described herein. Moreover the techniques can be used in combination with each other, as one of ordinary skill in the art using the techniques described herein how to combine the different aspects of the invention. All of these methods and combinations thereof can optionally include the use of mass-modified nucleotides and internal calibrants.

KITS

The present invention also includes kits for preparing nucleic acids for mass spectrometric analysis. In one embodiment, a kit comprises: a first primer capable of binding a first strand of one of said target nucleic acids at a region 5' to a region of interest of said target nucleic acid; a second primer capable of binding a second strand complementary to said first strand at a region 5' to said region of interest of said target nucleic acid; a DNA polymerase capable of extending said primers to form primer extension products of said first and second primers; and a restriction endonuclease capable of reducing length of amplified target nucleic acids. The restriction endonuclease can be a type II restriction endonuclease or a Type IIS. The first primer may be biotinylated, i.e. comprise at least one biotin. The kit may also comprise a solid support capable of selectively binding either a positive strand or a negative strand comprising the region of interest of an amplified target nucleic acids, and a matrix solution.

In another embodiment, a kit comprises a first primer capable of binding a first strand of one of said target nucleic acids at a region 5' to a region of interest of said target nucleic acid; a second primer capable of binding a second strand complementary to said first strand at a region 5' to said region of interest of said target nucleic acid; a DNA polymerase capable of extending said primers to form primer extension products of said first and second primers: wherein the first primer is cleavable by chemical or enzymatic treatment. The cleavable primers of the invention include those comprising a chemically cleavable site, an exonuclease blocking moiety, a Type IIS restriction endonuclease recognition site, or at least one biotin, but does not include a Type II restriction endonuclease recognition site where one of the complementary strands cannot be cleaved by said Type II restriction endonuclease. This kit may optionally comprise a solid support and a matrix solution.

The following examples are provided to illustrate embodiments of the invention, but do not limit the scope of the invention.

EXAMPLES

Example 1. PCR Amplification of a Single Target Nucleic Acid.

An example PCR protocol that may be employed in this invention is as follows. A sample containing 10-10,000 copies of a source DNA may be mixed with two antiparallel DNA primers that surround a target nucleic acid, *e.g.* the coding region for a gene involved in carcinogenesis. The target nucleic acid may be any sequence that is known or suspected to be polymorphic, including STRs, SSLP, and genetic deletions, insertions, or point polymorphisms. The PCR mix may typically be composed of: 8 µl 2.5 mM deoxynucleoside triphosphates, 10 µl 10X PCR buffer, 10 µl 25 mM $MgCl_2$, 3 µl 10 µM forward primer, 3 µl 10 µM reverse primer, 0.3 µl thermostable Taq DNA polymerase, 64.7 µl H_20, and 1 µl source DNA. The sample tube may then be sealed and placed into a thermal cycling device. A typical cycling protocol is as follows:

Step 1	95°C 2 min.
Step 2	95°C 15 sec.
Step 3	55°C 15 sec.
Step 4	72°C 1 min.
Step 5	repeat Steps 2-4 35 times
Step 6	72°C 5 min.
Step 7	stop

Example 2. Production of Single-Stranded Nucleic Acids by Asymmetric PCR.

The basic PCR procedure of Example 1 can be modified in order to produce predominantly one of the two strands. These asymmetric procedures involve modifying the ratios of the two primers, a typical ratio is 10:1. These procedures are described in Molecular Cloning: A Laboratory Manual, Sambrook *et al.*, 1989 (incorporated by reference herein).

Example 3. Production of Single-Stranded DNA via Biotinylated PCR Products.

For the preparation and capturing of amplified target nucleic acids to a solid support, one of the two primers used in PCR amplification may be synthesized with a biotin moiety internally or at the 5' end of the oligonucleotide. Following a standard PCR, the double-stranded product can be bound to a solid-phase surface coated with streptavidin. For example, 10 pmol of double-stranded PCR product is mixed with 5 μl of 10 mg/ml paramagnetic streptavidin-coated beads in a binding/washing buffer of 2.0 M NaCl, 10 mM TrisCl, 1 mM EDTA, pH 8.0. The solution is incubated for 15 min. at room temperature with mixing. Following incubation the tube is placed next to a high field, rare earth magnet and the paramagnetic beads with the bound biotinylated PCR product are precipitated to the wall of the tube. The supernatant is removed, and the particles, outside the influence of the magnetic field, are resuspended into binding/washing buffer. The beads and wash solution are mixed and then subjected once again to the magnetic field to precipitate the magnetic particles. The supernatant is once again removed and either the wash step is repeated or the alkaline denaturation step commences. To release the unbiotinylated strand from the double-stranded product, the beads are mixed with an alkaline denaturation solution such as, 0.1 M NaOH. The beads are incubated at room temperature for 10 min. which denatures the PCR product and releases the unbiotinylated product into solution. The biotinylated strand, bound to the magnetic beads is precipitated from the solution under the magnetic field and unbiotinylated strand, now single-stranded, can optionally be transferred to a new tube with the supernatant and readied for mass spectrometric analysis.

The bound single-stranded amplified target nucleic acid can be released from the streptavidin-coated beads using one of a number of different procedures. These procedures include denaturation of biotin/streptavidin bond by heat denaturation (95 °C for 5 min.), or the use of a denaturant, such as NaOH (1 mM NaOH for 15 min. at 65°C), and use of a secondary cleavable site, such as a disulfide linkage (100 mM DTT (dithiothreitol) for 15 min. at room temperature) or a 5' thiolated nucleotide (0.1 mM $AgNO_3$ for 15 min. at room temperature) present within the primer.

Example 4. Mass Modification of Target Nucleic Acids.

Mass modification of the target nucleic acid is typically performed during the amplification step. One or more standard deoxynucleoside triphosphates are replaced with modified deoxynucleoside triphosphates. As an example thymidine may be replaced with a 5-

alkynyl-substituted-2'-deoxyuridine triphosphate. Because the modified nucleotides may not be efficient substrates for DNA polymerase it may be necessary to increase the concentration of the corresponding triphosphate by a factor of 2 to 100 over normal levels.

Example 5. Analysis of Single-Stranded Amplified Tetranucleotide Repeat Region of THO1 Gene.

A sample of human genomic DNA is subjected to PCR amplification with the primer pair: (SEQ ID NO: 2) 5'-Biotin-GTGATTCCCATTGGCCTGT(sT)CCTC-3' and (SEQ ID NO:2) 5'- AGTGCAGGTCACAGGGAACACAGA-3', which selectively amplify the tetranucleotide repeat region of the tyrosine hydroxylase ("THO1") gene to give 90-114 bp PCR products where sT is 5'-thiolated thymidine, also known as 2'-deoxythymidine-5'-(S)-phosphorothioate (see FIG. 16). These PCR amplified target nucleic acids are about a factor of 2 smaller products than current commercially available primers provide. The PCR reaction is performed on a 50 μL scale using 17.5 pmol of each primer and 50-100 ng of template, with 30 3-step thermal cycles, following an initial step at 94°C for 2 min, of 94°C for 45 s followed by 55°C for 30 s and then 72°C for 30 s. A final step at 72°C for 5 min is added to complete the reaction. The biotinylated product is bound to streptavidin-coated magnetic beads MPG (CPG Inc., Great Neck, NY) and then subjected to denaturation conditions of 0.1 M NaOH for 10 minutes. The solid-support-bound single-stranded target nucleic acid is then subjected to extensive washing with 10 mM ammonium acetate and deionized water. The single-stranded amplified target nucleic acid is then released from the beads by cleavage of the P-S bond in the cleavable primer with 0.1 mM $AgNO_3$. After 2 μL of 100 mM dithiothreitol is added to sequester the Ag^+ ion, the sample is evaporated to dryness in a Speed-vac concentrator. For analysis, the sample is redissolved in 1 μL deionized H_20 and is mixed with 1 μL of matrix solution consisting of 3-HPA (3-hydroxypicolinic acid) in acetonitrile:H_2O 1:1. The sample is deposited onto a silicon stage, dried under a gentle flow of nitrogen and is placed into the mass spectrometer.

The experimental apparatus used for analyzing the sample amplified target nucleic acids is composed of an excitation source, a sample manipulator and a TOF mass spectrometer. The excitation source used for desorption is a Nd:YAG laser. The laser is operated at a 10 Hz repetition rate, with a 5 nanosecond pulse width. The desorption laser beam maintained at an incident angle of 45° is focused onto the sample with a 250 mm focal length AL-2 coated spherical mirror to an elliptical spot size of approximately 100 by 150 μm. A Clan-laser

polarizer (Newport Corporation, Fountain Valley, CA) is placed in a rotation stage in the beam path for continuously variable attenuation, allowing adjustment of the polarized Nd:YAG laser energy density from below 1 mJ/cm^2 to 100 mJ/cm^2. The optimum energy density for desorption is in the range of 10 to 20 mJ/cm^2.

Mass spectra are recorded in positive-ion mode at room temperature. The sample region is evacuated by a 300 liter per second turbomolecular pump. The drift and detection regions are evacuated using a cryopump with nominal 1500 liter per second pumping speed. The base pressure of the chamber is 3×10^{-9} Torr, and the normal working pressure, within about five minutes of sample introduction, is 5×10^{-8} Torr. A total of 100 laser shots are summed to obtain a spectrum. The spectrum shown in FIG. 13 reveals two clear peaks corresponding to an 84-mer and a 91-mer, which are the expected product sizes corresponding to 8 and 9.3 repeats with one extra adenine base added to the 3'-end of each, due to the well-known property of *Taq* DNA polymerase to yield one-base over-extensions. The two peaks arise because the final sample, though single-stranded, derives from amplification of a heterozygous allele.

Example 6. Comparison of mass spectra for a single-stranded amplified THO1 ladder of nucleic acids and a single-stranded amplified THO1 ladder that have had their lengths reduced by endonuclease cleavage.

A 1 μL sample of TH01 ladder (Promega Inc., Madison, WI) which contains PCR products ranging in size from 179-203 bp is reamplified in a 100 μL reaction volume according to the same amplification protocol as described above for genomic DNA to yield a ladder of products in the size range of 90-114 bp. One half of the product mixture (amplified target nucleic acids) is bound to streptavidin-coated magnetic beads, denatured, washed and cleaved from the beads as described in the previous example. The other half of the product mixture is then bound to the streptavidin-coated magnetic beads and washed. Then, 2 μL of 10X NEB Buffer 4 is added to the second half of the amplified target nucleic acids, followed by the addition of 16 μL of H_20 and 20 units of *Nco* I restriction endonuclease (New England Biolabs, Beverly, MA) recognizing CCATGG. This restriction endonuclease was chosen to reduce the length of the amplified target nucleic acid because the amplified target nucleic acid contained a Nco I recognition site (in the wild type or consensus sequence) which was located in a flanking region and not in the region of interest. The mixture is incubated at 37°C for 1 hour after which the enzyme is washed away, the amplified target nucleic acids are bound through biotinylated

primers, denatured, washed and single-stranded amplified target nucleic acids are then cleaved from the beads with $AgNO_3$. The restriction enzyme-digested ladder has a size range of 40 to 64 bases as a result of the enzyme cutting 3 bp past the end of the final CATT repeat. Samples of both undigested/full-length and digested/reduced-length products are prepared for mass spectrometry analysis by reducing the volume by evaporation and adding 1 μL of 3-hydroxypicolinic acid matrix solution and allowing to dry on the sample plate. The resulting positive-ion mass spectra of the undigested and digested ladders are shown in FIG. 14 and FIG. 15, respectively. The mass resolution of the peaks for the digested ladder (FIG. 15) is much greater than that for the undigested ladder (FIG. 14). This is due to the use of Nco I to reduce the length of the single-stranded amplified target nucleic acids that were subjected to mass spectrometric analysis.

Example 7. Mass Spectrometry Analysis.

The single-stranded amplified target nucleic acid sample to be analyzed is typically mixed with an equal volume of matrix solution consisting of 0.5 M 3-hydroxypicolinic acid (3-HPA) and 50 mM diammonium hydrogen citrate. Typically, a 1 μL portion of the sample is applied to the mass spectrometer sample stage and allowed to dry under a gentle stream of nitrogen gas at room temperature. When the sample has completely dried to form crystals (typically 5 min.) the sample is inserted into the mass spectrometer for analysis. The usual analysis conditions employ the use of a Nd:YAG laser operating at 266 nm with an average pulse energy of 15 mJ/cm^2. An average of 100 laser shots is typically used to obtain a spectrum.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the invention and the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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Youil *et al.*, "Screening for Mutations by Enzyme Mismatch Cleavage with T4 Endonuclease VII," *Proc. Natl. Acad. Sci. USA*, 92:87, 1995.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: METHODS OF PREPARING NUCLEIC ACIDS FOR MASS SPECTROMETRIC ANALYSIS

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/759,993
(B) FILING DATE: 02-DEC-1996

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/032,369
(B) FILING DATE: 02-DEC-1996

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/715,582
(B) FILING DATE: 19-SEP-1996

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION:1
(D) OTHER INFORMATION:/note= "5' end is biotinylated"

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:20
(D) OTHER INFORMATION:/note= "5'-thio-thymidine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GTGATTCCCA TTGGCCTGTT CCTC 24

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AGTGCAGGTC ACAGGGAACA CAGA 24

CLAIMS

1. A method of determining the mass of a target nucleic acid by mass spectrometric analysis comprising:

a) identifying a target nucleic acid, wherein the target nucleic acid comprises a region of interest and one or more flanking regions;

b) reducing the length of the target nucleic acid by cleaving at least a portion of one or more of said flanking regions to produce a reduced-length target nucleic acid;

c) obtaining a single-stranded reduced-length target nucleic acid; and

d) determining the mass of the single-stranded reduced-length target nucleic acid using a mass spectrometer.

2. The method of claim 1, further comprising amplifying the target nucleic acid prior to reducing the length of the target nucleic acid to produce an amplified target nucleic acid.

3. The method of claim 2, wherein the reducing step comprises using a restriction endonuclease capable of cleaving at a cleavable site.

4. The method of claim 3, wherein the restriction endonuclease is a Type IIS restriction endonuclease.

5. The method of claim 3, wherein the restriction endonuclease is a Type II restriction endonuclease.

6. The method of claim 3, wherein the amplifying step comprises using a cleavable primer comprising a recognition site for the restriction endonuclease.

7. The method of claim 2, wherein the amplifying step comprises using a cleavable primer comprising a cleavable site.

8. The method of claim 7, wherein the reducing step comprises treating the amplified target nucleic acids with a 5' to 3' exonuclease.

9. The method of claim 7, wherein the reducing step comprises cleaving at a chemically cleavable site.

10. The method of claim 9, wherein the chemically cleavable site comprises a modified base, a modified sugar, or a chemically cleavable group incorporated into the phosphate backbone.

11. The method of claim 10, wherein the chemically cleavable site comprises a chemically cleavable group.

12. The method of claim 11, wherein the chemically cleavable group comprises dialkoxysilane, 3'-(S)-phosphorothioate, 5'-(S)-phosphorothioate, 3'-(N)-phosphoroamidate, or 5'-(N)-phosphoroamidate.

13. The method of claim 12, wherein the chemically cleavable group comprises 3'-(S)-phosphorothioate or 5'-(S)-phosphorothioate.

14. The method of claim 10, wherein the chemically cleavable site comprises a modified sugar.

15. The method of claim 14, wherein the modified sugar comprises ribose.

16. The method of claim 2, wherein the amplified target nucleic acid further comprises a first strand and a second complementary strand and the obtaining step comprises:

a) attaching the first strand of the amplified target nucleic acid to a solid support; and

b) separating the first strand from the second strand to produce a bound first strand and an unbound second strand, wherein the mass of the unbound second strand is determined using a mass spectrometer.

17. The method of claim 16, wherein the reducing step comprises using a restriction endonuclease capable of cleaving at a cleavable site.

18. The method of claim 17, wherein the restriction endonuclease is a Type IIS restriction endonuclease.

19. The method of claim 17, wherein the restriction endonuclease is a Type II restriction endonuclease.

20. The method of claim 17, wherein the amplifying step further comprises using a cleavable primer comprising a recognition site for the restriction endonuclease.

21. The method of claim 16, wherein the amplifying step further comprises using a cleavable primer comprising a cleavable site.

22. The method of claim 21, wherein the reducing step comprises treating the amplified target nucleic acids with a 5' to 3' exonuclease.

23. The method of claim 21, wherein the cleavable site comprises a chemically cleavable site.

24. The method of claim 23, wherein the chemically cleavable site comprises a modified base, a modified sugar, or a chemically cleavable group incorporated into the phosphate backbone.

25. The method of claim 24, wherein the chemically cleavable site comprises a chemically cleavable group.

26. The method of claim 25, wherein the chemically cleavable group comprises dialkoxysilane, 3'-(S)-phosphorothioate, 5'-(S)-phosphorothioate, 3'-(N)-phosphoroamidate, or 5'-(N)-phosphoroamidate.

27. The method of claim 26, wherein the chemically cleavable group comprises 3'-(S)-phosphorothioate or 5'-(S)-phosphorothioate.

28. The method of claim 27, wherein the first strand is biotinylated and, wherein the solid support contains avidin or streptavidin.

29. The method of claim 28 wherein the amplified target nucleic acid comprises a mass-modified nucleotide.

30. The method of claim 25, wherein the chemically cleavable site comprises a modified sugar.

31. The method of claim 30, wherein the modified sugar comprises ribose.

32. The method of claim 16, wherein the first strand is biotinylated and, wherein the solid support contains avidin or streptavidin.

33. The method of claim 16, wherein the amplified target nucleic acid comprises a mass-modified nucleotide.

34. The method of claim 16, wherein said determining step further comprises utilizing internal self-calibrants.

35. The method of claim 2, wherein the amplified target nucleic acid comprises a first strand and a second complementary strand and the obtaining step comprises:

a) attaching the first strand of the amplified target nucleic acid to a solid support;

b) separating the first strand from the second strand to produce a bound first strand and an unbound second strand;

c) removing the unbound second strand; and

d) releasing the bound first strand from the solid support to produce a single-stranded reduced-length amplified target nucleic acid for mass spectral analysis.

36. The method of claim 35, wherein the releasing step comprises using a cleavable linker.

37. The method of claim 35, wherein the reducing step comprises using a restriction endonuclease capable of cleaving at a cleavable site.

38. The method of claim 37, wherein the restriction endonuclease is a Type IIS restriction endonuclease.

39. The method of claim 37, wherein the restriction endonuclease is a Type II restriction endonuclease.

40. The method of claim 37, wherein the amplifying step further comprises using a cleavable primer comprising a recognition site for the restriction endonuclease.

41. The method of claim 35, wherein the amplifying step further comprises using a cleavable primer comprising a cleavable site.

42. The method of claim 41, wherein the reducing step comprises treating the amplified target nucleic acids with a 5' to 3' exonuclease.

43. The method of claim 41, wherein the cleavable site comprises a chemically cleavable site.

44. The method of claim 43, wherein the chemically cleavable site comprises a modified base, a modified sugar, or a chemically cleavable group incorporated into the phosphate backbone.

45. The method of claim 44, wherein the chemically cleavable site comprises a chemically cleavable group.

46. The method of claim 45, wherein the chemically cleavable group comprises dialkoxysilane, 3'-(S)-phosphorothioate, 5'-(S)-phosphorothioate, 3'-(N)-phosphoroamidate, or 5'-(N)-phosphoroamidate.

47. The method of claim 46, wherein the chemically cleavable group comprises 3'-(S)-phosphorothioate or 5'-(S)-phosphorothioate.

48. The method of claim 47, wherein the first strand is biotinylated and, wherein the solid support contains avidin or streptavidin.

49. The method of claim 48, wherein the amplified target nucleic acid comprises a mass-modified nucleotide.

50. The method of claim 44, wherein the chemically cleavable site comprises a modified sugar.

51. The method of claim 50, wherein the modified sugar comprises ribose.

52. The method of claim 35, wherein the first strand is biotinylated and, wherein the solid support contains avidin or streptavidin.

53. The method of claim 35, wherein the amplified target nucleic acid comprises a mass-modified nucleotide.

54. The method of claim 35, wherein said determining step further comprises utilizing internal self-calibrants.

55. A method of preparing a double-stranded target nucleic acid for mass spectrometric analysis comprising:

a) amplifying a target nucleic acid to produce an amplified target nucleic acid, wherein the amplified target nucleic acid comprises a first strand and a second complementary strand;

b) attaching the first strand of the amplified target nucleic acid to a solid support to produce a bound first strand and an unbound second strand;

c) removing the unbound second strand from the bound first strand;

d) releasing the bound first strand from the solid support to form a single-stranded amplified target nucleic acid; and

e) determining the mass of the single-stranded amplified target nucleic acid using a mass spectrometer.

56. The method of claim 55, wherein the releasing step comprises using a cleavable linker.

57. The method of claim 56, wherein said determining does not involve sequencing of the amplified target nucleic acid.

58. The method of claim 55, wherein the releasing step comprises using a restriction endonuclease capable of cleaving at a cleavable site.

59. The method of claim 55, wherein the restriction endonuclease is a Type IIS restriction endonuclease.

60. The method of claim 59, wherein the restriction endonuclease is a Type II restriction endonuclease.

61. The method of claim 58, wherein the amplifying step further comprises using a cleavable primer comprising a recognition site for the restriction endonuclease.

62. The method of claim 55, wherein the amplifying step further comprises using a cleavable primer comprising a cleavable site.

63. The method of claim 62, wherein the releasing step comprises treating the amplified target nucleic acids with a 5' to 3' exonuclease.

64. The method of claim 62, wherein the releasing step comprises cleaving at a chemically cleavable site.

65. The method of claim 64, wherein the chemically cleavable site comprises a modified base, a modified sugar, or a chemically cleavable group incorporated into the phosphate backbone.

66. The method of claim 65, wherein the chemically cleavable site comprises a chemically cleavable group.

67. The method of claim 66, wherein the chemically cleavable group comprises dialkoxysilane, 3'-(S)-phosphorothioate, 5'-(S)-phosphorothioate, 3'-(N)-phosphoroamidate, or 5'-(N)-phosphoroamidate.

68. The method of claim 67, wherein the chemically cleavable group comprises 3'-(S)-phosphorothioate or 5'-(S)-phosphorothioate.

69. The method of claim 69, wherein the first strand is biotinylated and, wherein the solid support contains avidin or streptavidin.

70. The method of claim 69, wherein the amplified target nucleic acid comprises a mass-modified nucleotide.

71. The method of claim 65, wherein the chemically cleavable site comprises a modified sugar.

72. The method of claim 71, wherein the modified sugar comprises ribose.

73. The method of claim 55, wherein the first strand is biotinylated and, wherein the solid support contains avidin or streptavidin.

74. The method of claim 55, wherein the amplified target nucleic acid comprises a mass-modified nucleotide.

75. The method of claim 55, wherein said determining step further comprises utilizing internal self-calibrants.

76. A kit for preparing a double-stranded target nucleic acid having a first strand and a second complementary strand for mass spectrometric analysis comprising:

a) a first primer capable of binding the first strand of the target nucleic acid 5' to a region of interest of the target nucleic acid;

b) a second primer capable of binding the second strand of the target nucleic acid 5' to the region of interest of the target nucleic acid;

c) a DNA polymerase; and

d) a restriction endonuclease.

77. The kit of claim 76, wherein the restriction endonuclease is a Type II restriction endonuclease.

78. The kit of claim 76, wherein the restriction endonuclease is a Type IIS restriction endonuclease.

79. The kit of claim 76, wherein the first primer is biotinylated.

80. The kit of claim 76, further comprising a solid support capable of selectively binding the first strand of the target nucleic acid.

81. The kit of claim 76, further comprising a matrix.

82. The kit of claim 81, wherein the matrix comprises 3-hydroxypicolinic acid.

83. A kit for preparing a double-stranded target nucleic acid having a first strand and a second complementary strand for mass spectrometric analysis comprising:

a) a first primer capable of binding the first strand of the target nucleic acid 5' to a region of interest of the target nucleic acid, wherein said first primer comprises a cleavable primer;

b) a second primer capable of binding the second strand 5' to the region of interest of the target nucleic acid; and

c) a DNA polymerase.

84. The kit of claim 83, wherein the cleavable primer comprises a chemically cleavable site.

85. The kit of claim 84, wherein the chemically cleavable site comprises a modified base, a modified sugar, or a chemically cleavable group incorporated into the phosphate backbone.

86. The method of claim 85, wherein the chemically cleavable site comprises a chemically cleavable group.

87. The kit of claim 86, wherein the chemically cleavable group comprises dialkoxysilane, 3'-(S)-phosphorothioate, 5'-(S)-phosphorothioate, 3'-(N)-phosphoroamidate, or 5'-(N)-phosphoroamidate.

88. The kit of claim 87, wherein the chemically cleavable group comprises 3'-(S)-phosphorothioate or 5'-(S)-phosphorothioate.

89. The kit of claim 83, wherein the cleavable primer comprises an exonuclease blocking moiety.

90. The kit of claim 83, wherein the cleavable primer comprises a Type IIS restriction endonuclease recognition site.

91. The kit of claim 83 further comprising a solid support capable of selectively binding the first strand of the target nucleic acid.

92. The kit of claim 91, wherein the first strand is biotinylated.

93. The kit of claim 83, further comprising a matrix.

94. The kit of claim 93, wherein the matrix comprises 3-hydroxypicolinic acid.

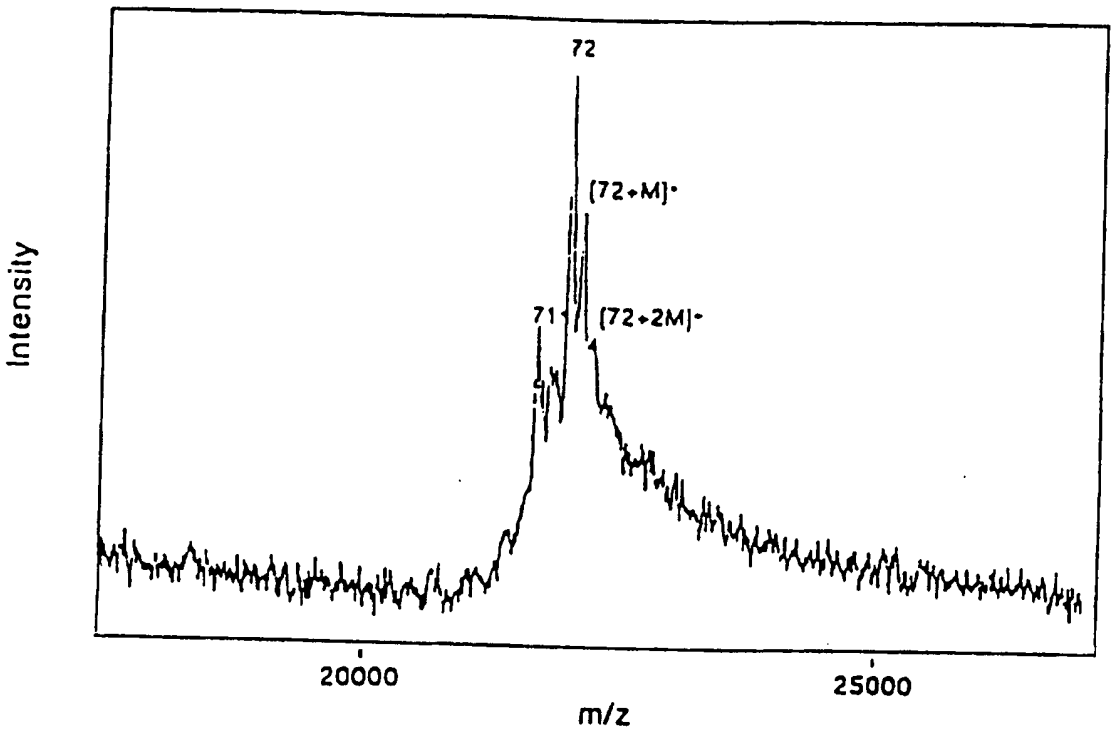

FIG. 1A

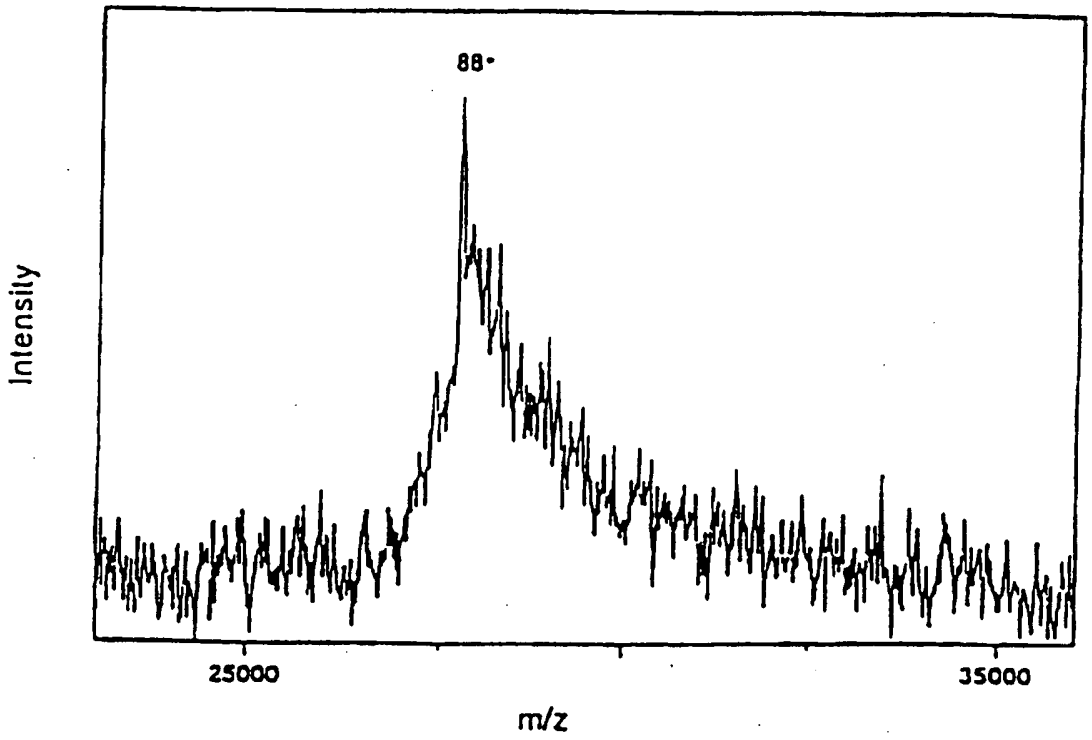

FIG. 1B

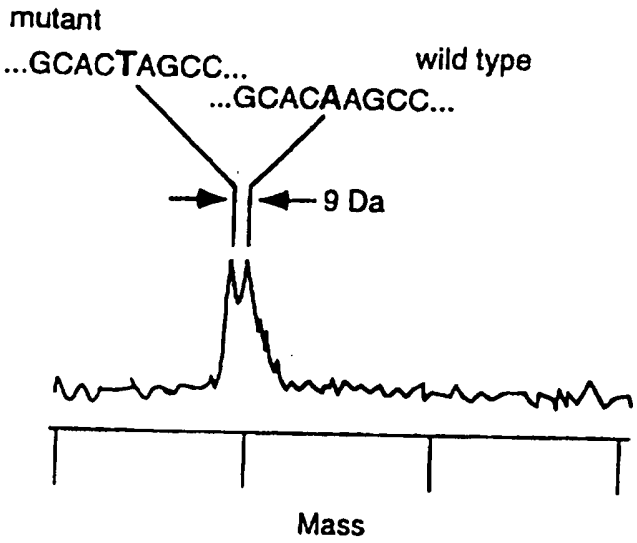

FIG. 2A

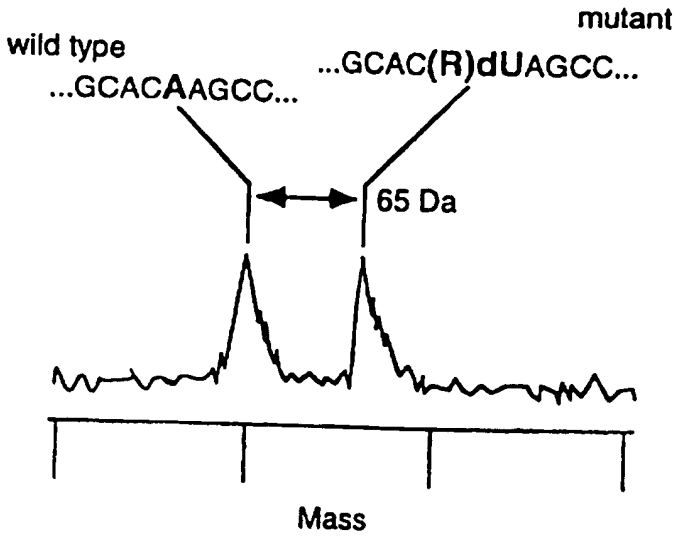

FIG. 2B

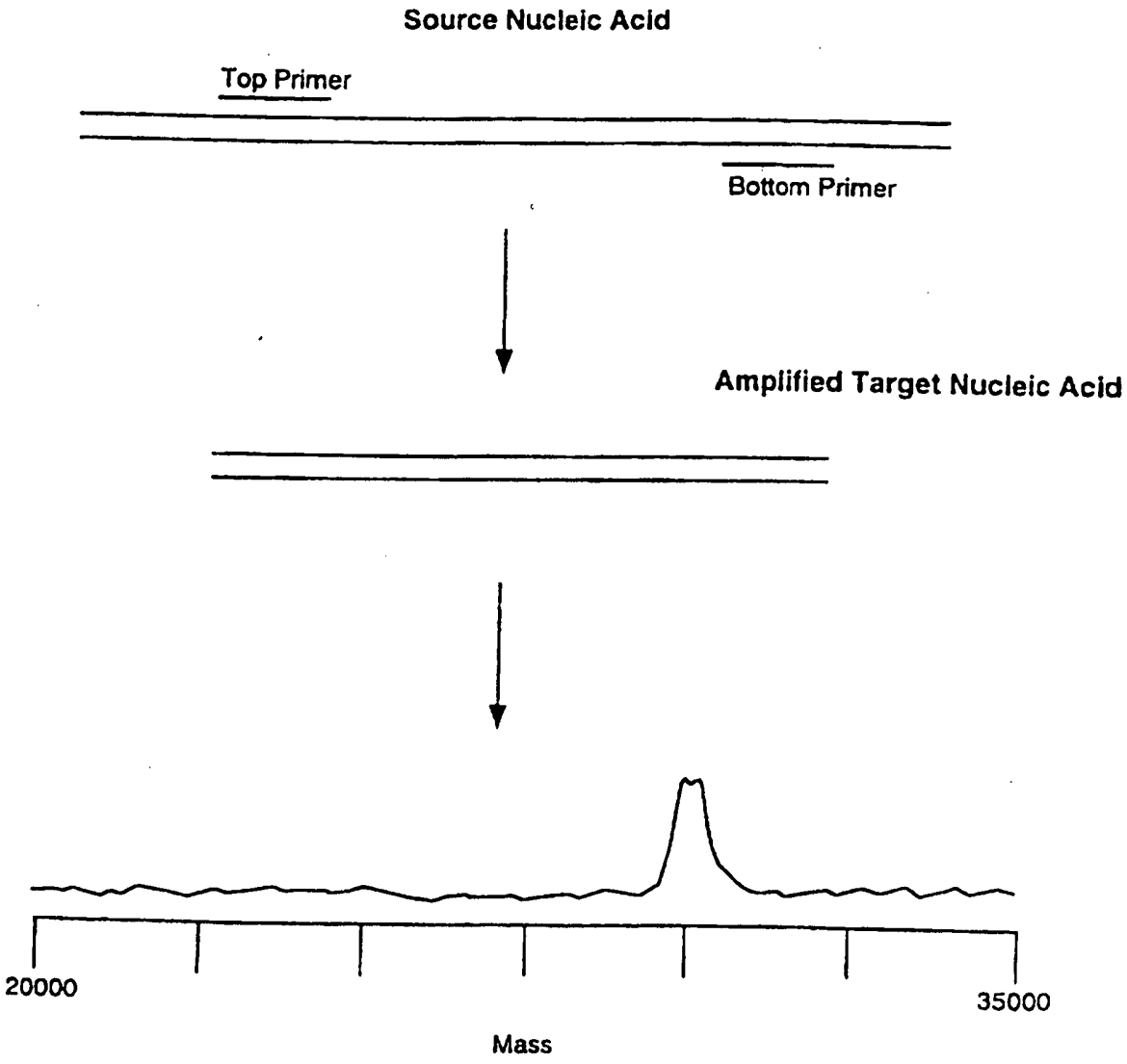
Source Nucleic Acid
Top Primer
Bottom Primer
Amplified Target Nucleic Acid
20000
35000
Mass

FIG. 3

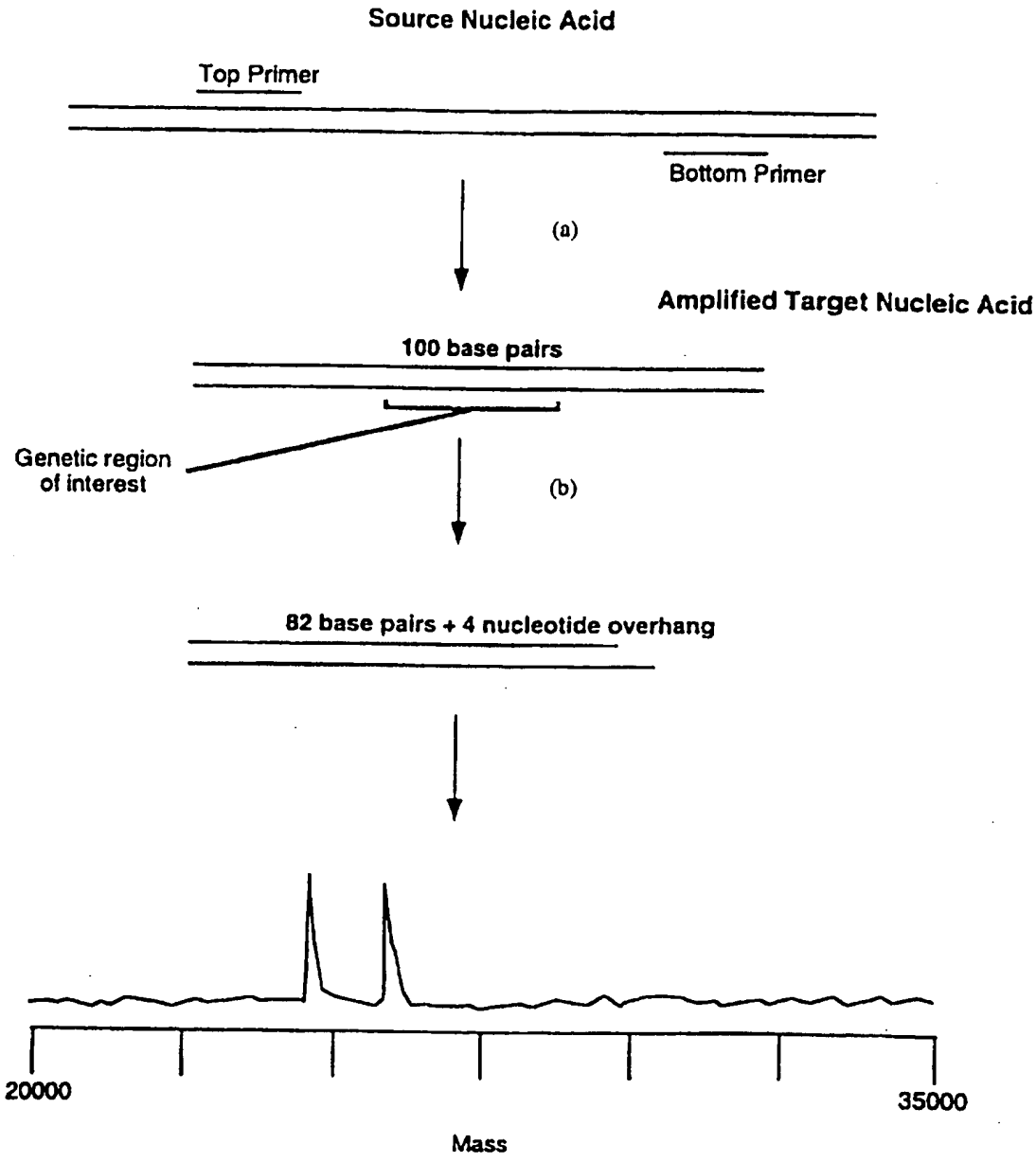
Source Nucleic Acid
Top Primer
Bottom Primer
(a)
Amplified Target Nucleic Acid
100 base pairs
Genetic region of interest
(b)
82 base pairs + 4 nucleotide overhang
20000
35000
Mass

FIG. 4

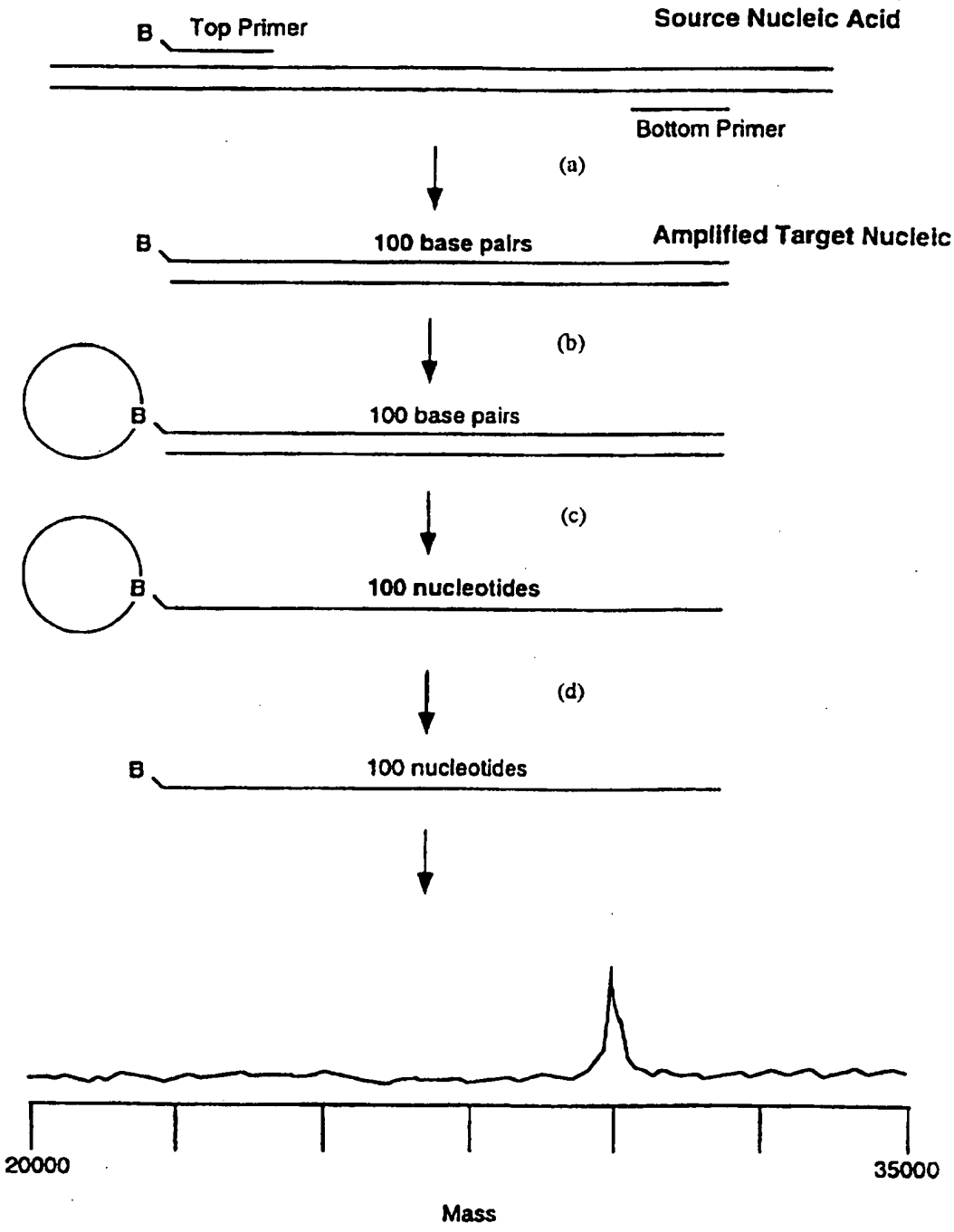
B
Top Primer
Source Nucleic Acid
Bottom Primer
(a)
B
100 base pairs
Amplified Target Nucleic Acid
(b)
B
100 base pairs
(c)
B
100 nucleotides
(d)
B
100 nucleotides
20000
35000
Mass

FIG. 5

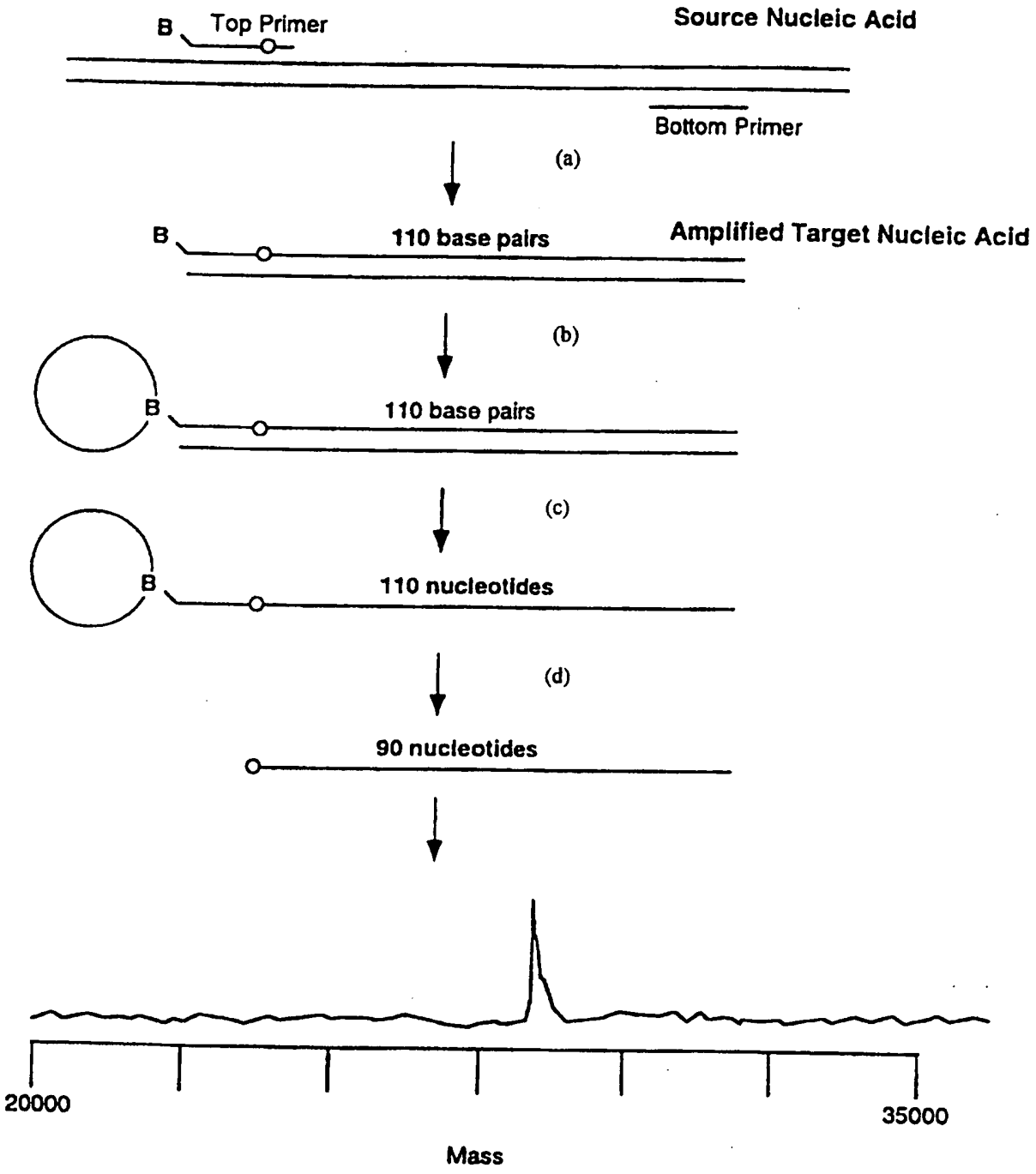
B
Top Primer
Source Nucleic Acid
Bottom Primer
(a)
B
110 base pairs
Amplified Target Nucleic Acid
(b)
B
110 base pairs
(c)
B
110 nucleotides
(d)
90 nucleotides
20000
35000
Mass

FIG. 6

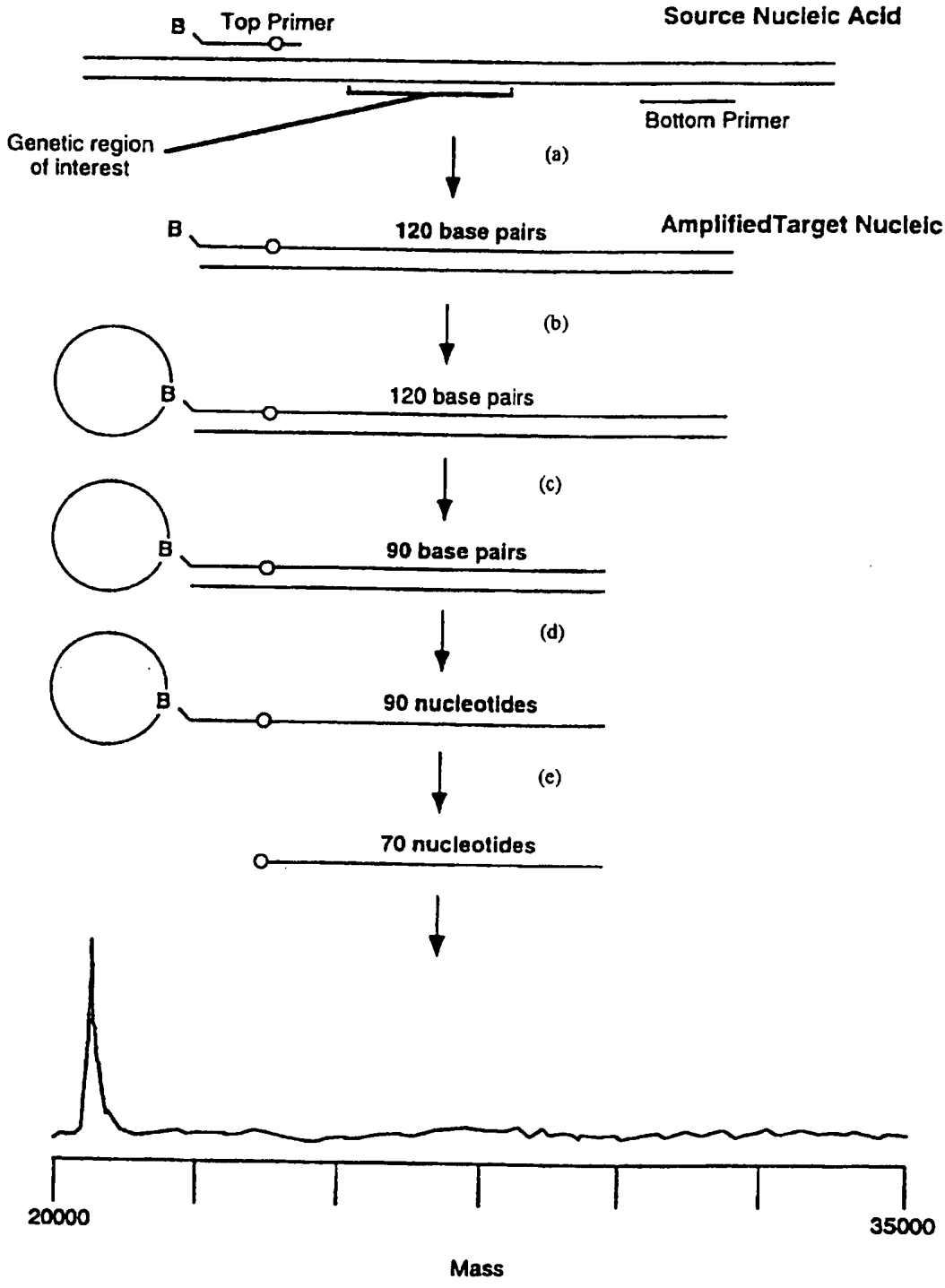
B
Top Primer
Source Nucleic Acid
Bottom Primer
Genetic region of interest
(a)
B
120 base pairs
AmplifiedTarget Nucleic Acid
(b)
B
120 base pairs
(c)
B
90 base pairs
(d)
B
90 nucleotides
(e)
70 nucleotides
20000
35000
Mass

FIG. 7

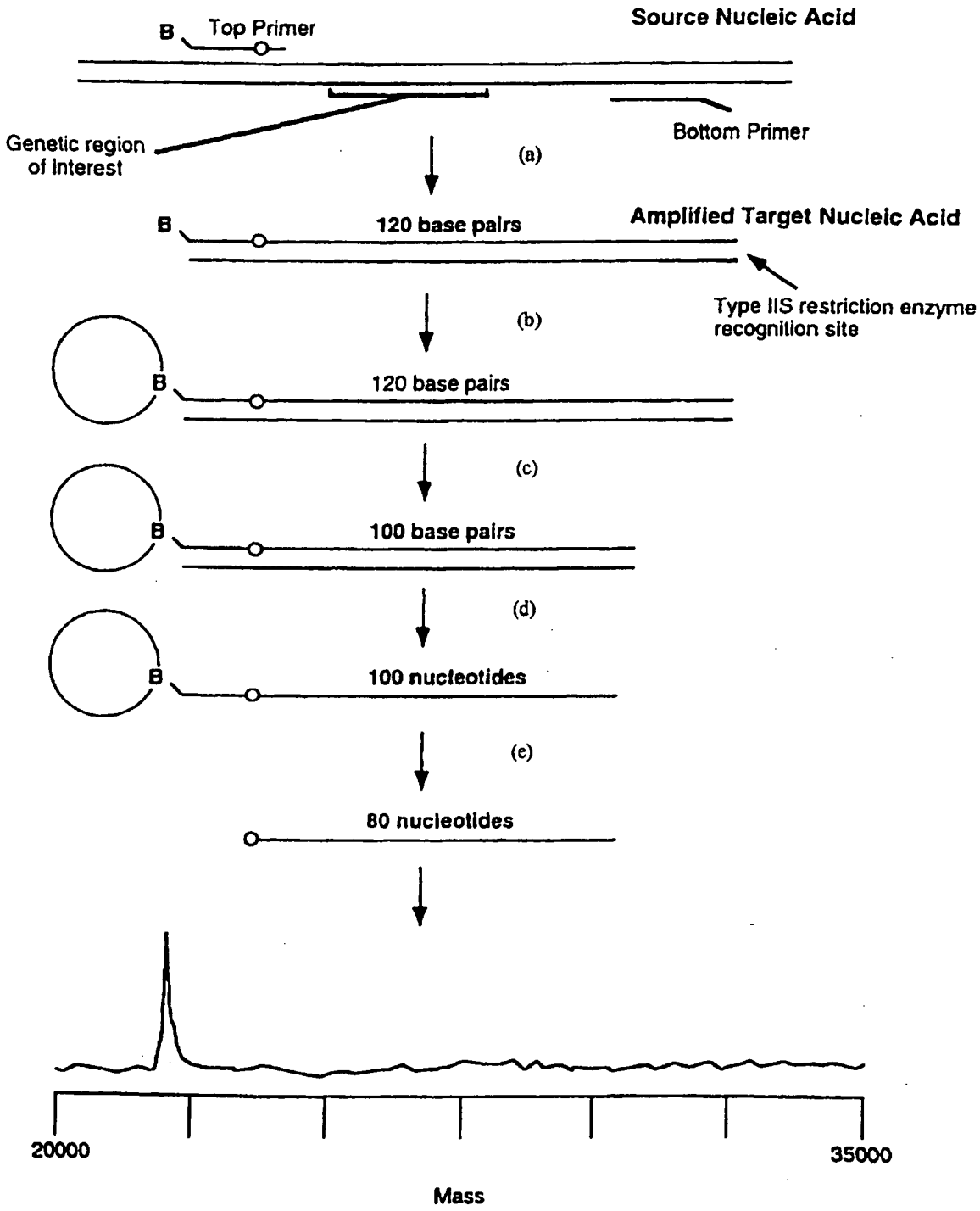
B
Top Primer
Source Nucleic Acid
Genetic region of interest
Bottom Primer
(a)
B
120 base pairs
Amplified Target Nucleic Acid
Type IIS restriction enzyme recognition site
(b)
B
120 base pairs
(c)
B
100 base pairs
(d)
B
100 nucleotides
(e)
80 nucleotides
20000
35000
Mass

FIG. 8

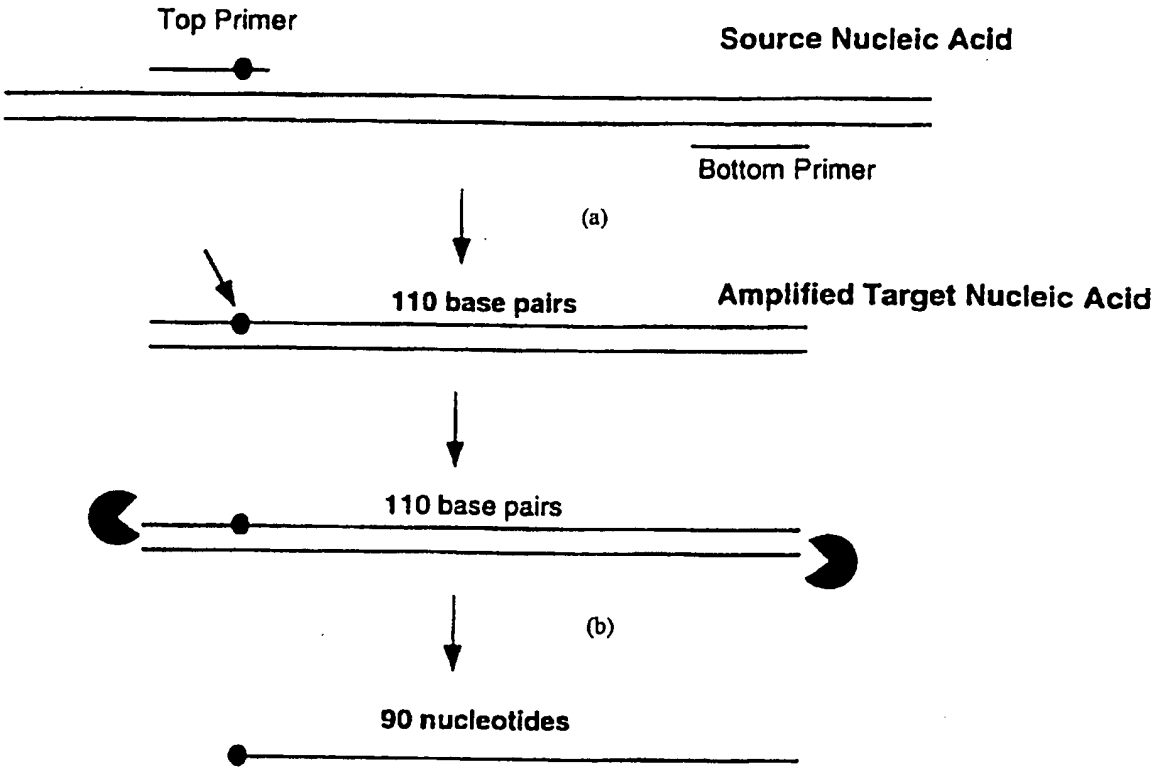
Top Primer
Source Nucleic Acid
Bottom Primer
(a)
110 base pairs
Amplified Target Nucleic Acid
110 base pairs
(b)
90 nucleotides

FIG. 9

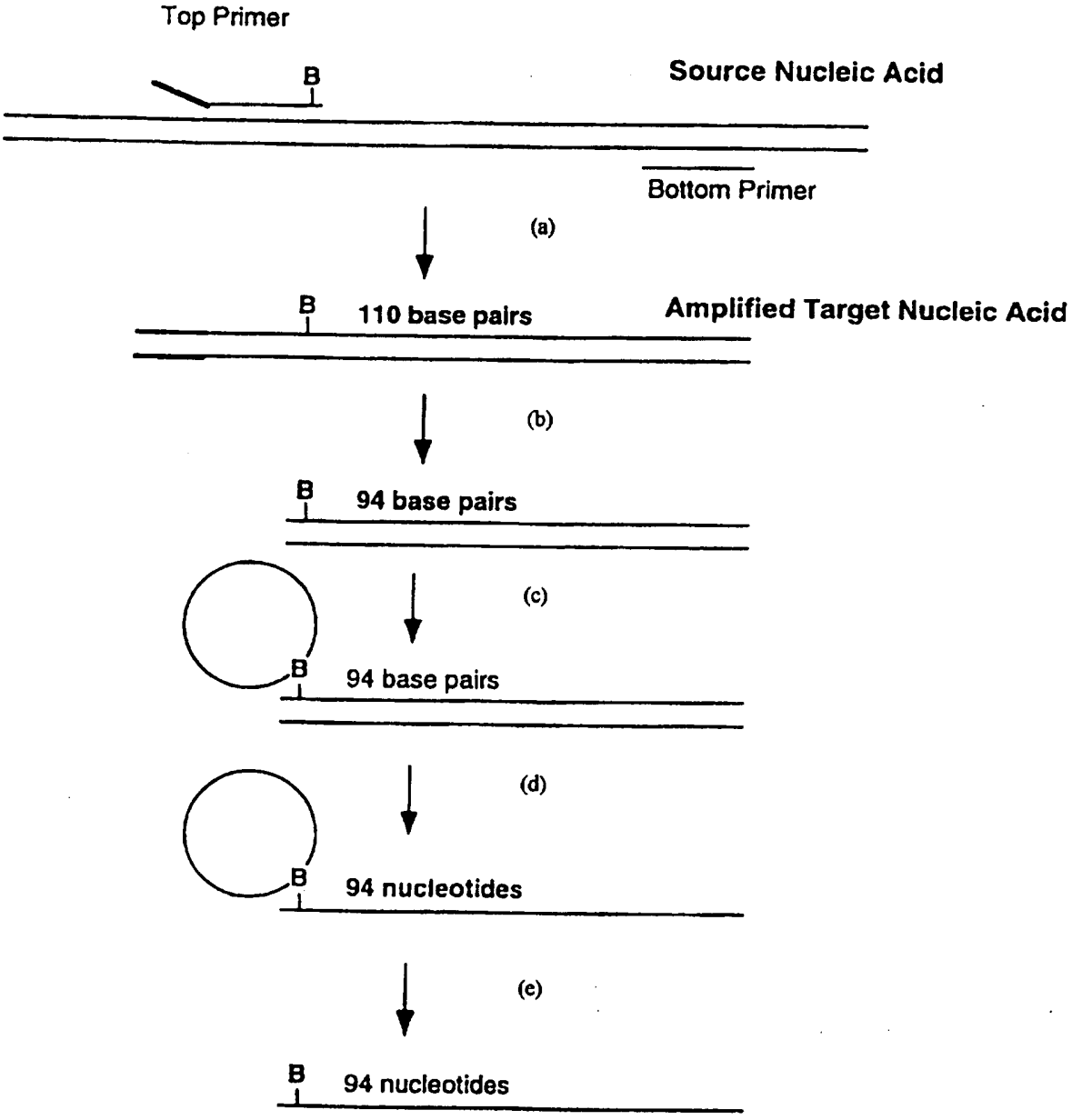
Top Primer
B
Source Nucleic Acid
Bottom Primer
(a)
B
110 base pairs
Amplified Target Nucleic Acid
(b)
B
94 base pairs
(c)
B
94 base pairs
(d)
B
94 nucleotides
(e)
B
94 nucleotides

FIG. 10

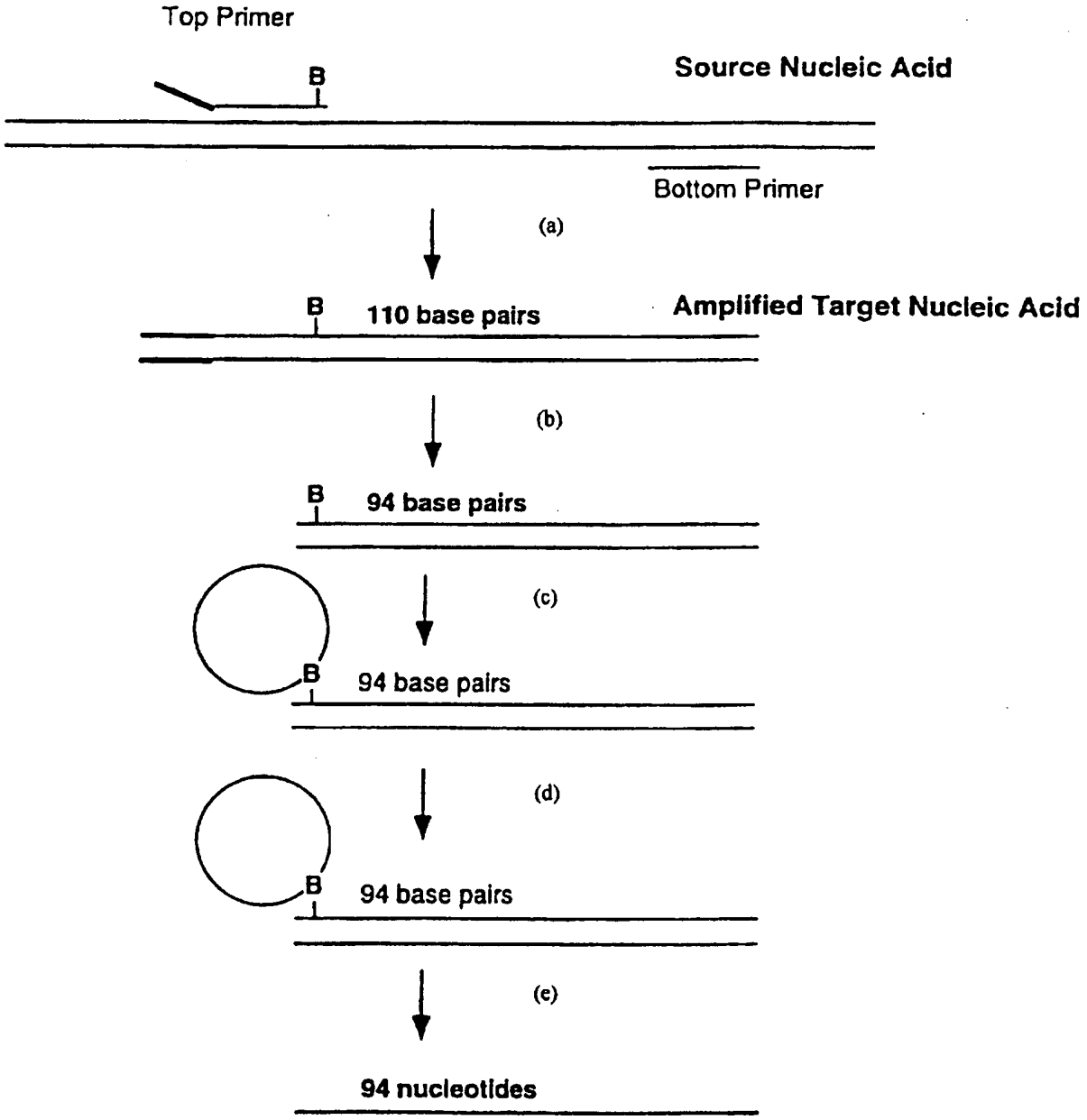
Top Primer
B
Source Nucleic Acid
Bottom Primer
(a)
B
110 base pairs
Amplified Target Nucleic Acid
(b)
B
94 base pairs
(c)
B
94 base pairs
(d)
B
94 base pairs
(e)
94 nucleotides

FIG. 11

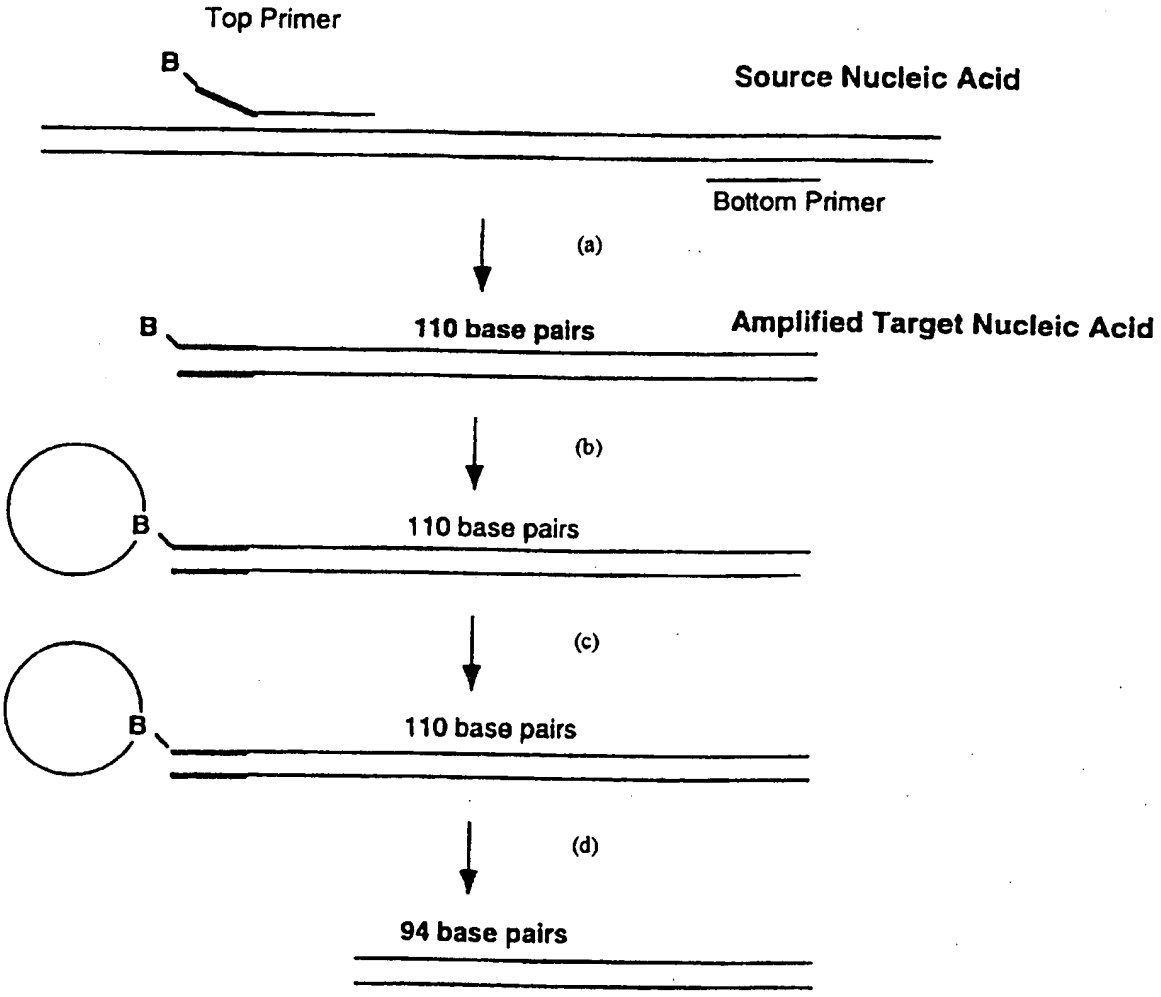
Top Primer
B
Source Nucleic Acid
Bottom Primer
(a)
B
110 base pairs
Amplified Target Nucleic Acid
(b)
B
110 base pairs
(c)
B
110 base pairs
(d)
94 base pairs

FIG. 12

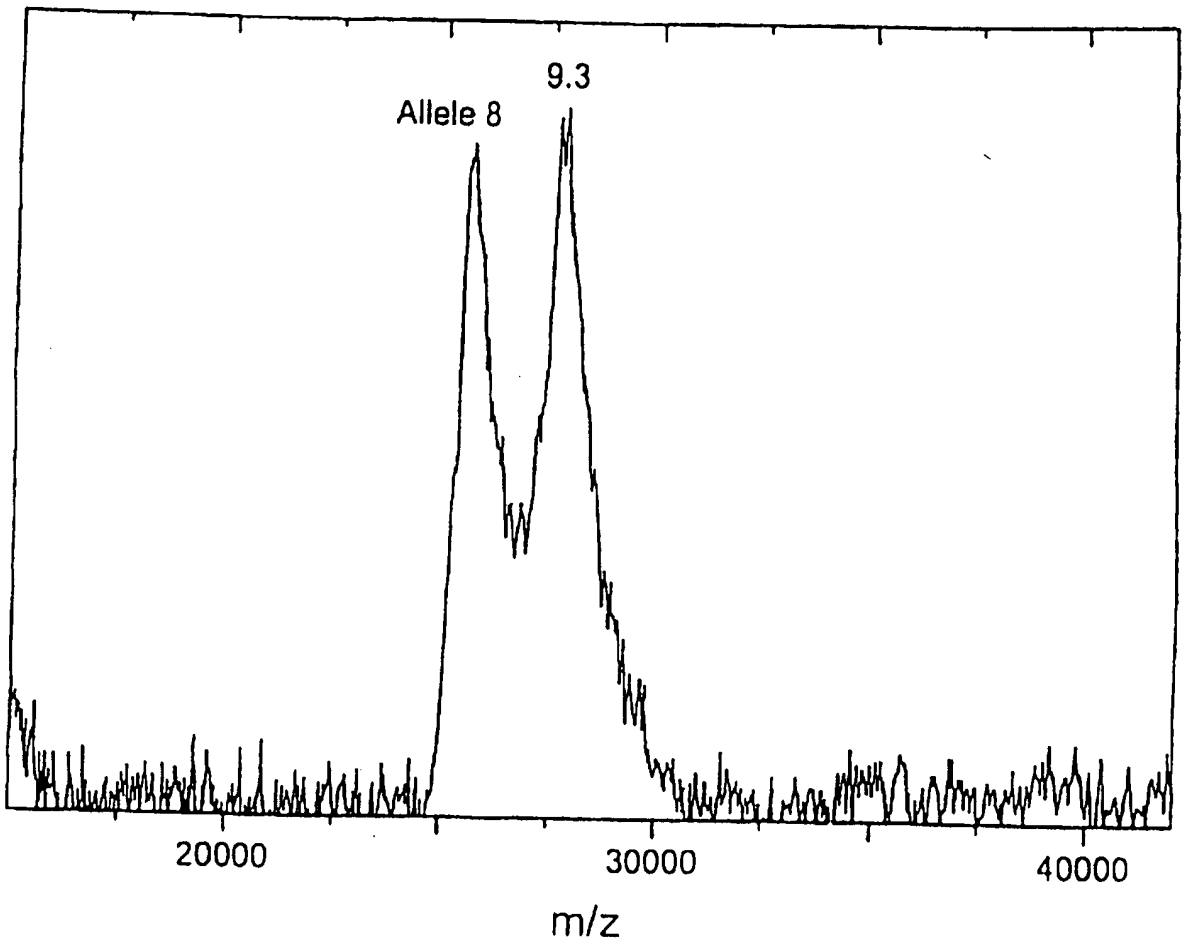

FIG. 13

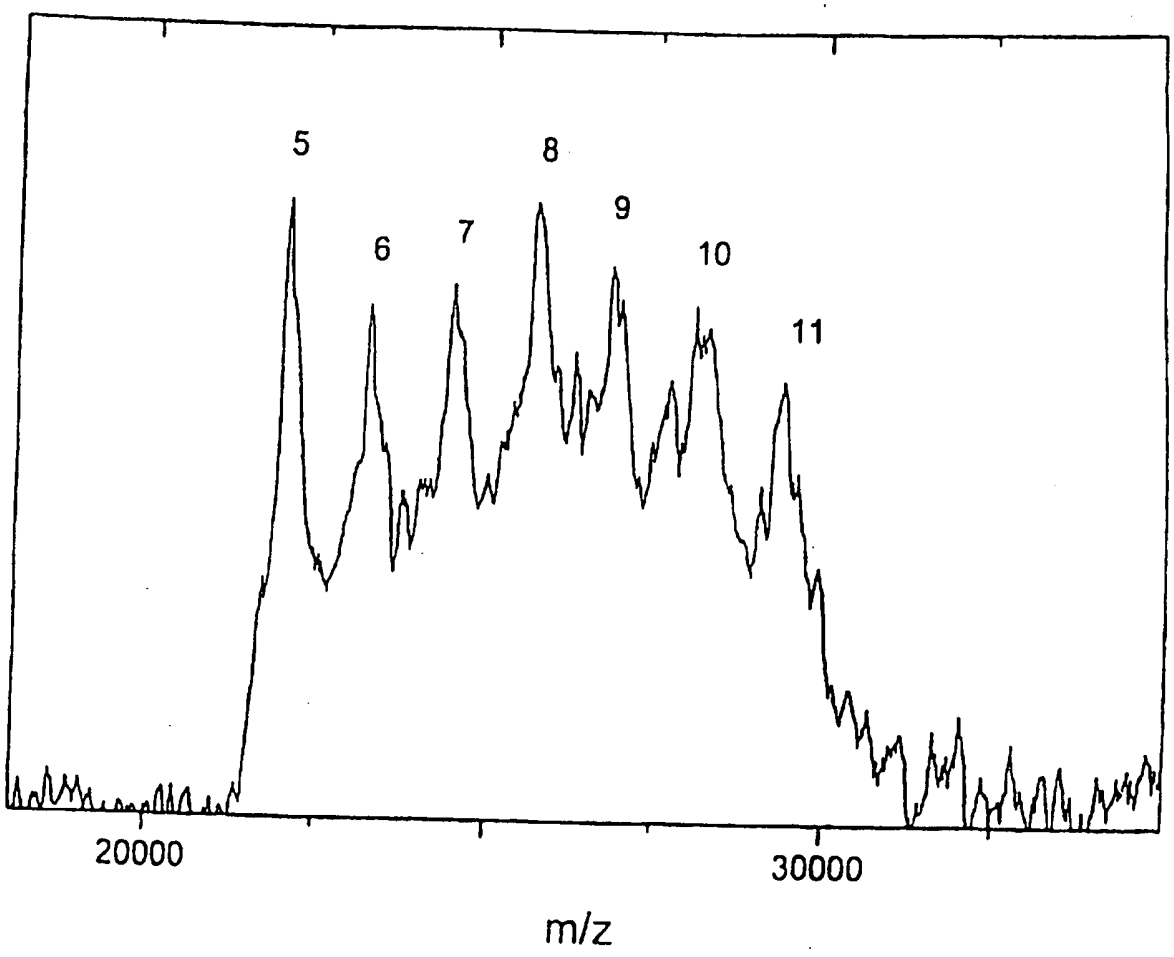

FIG. 14

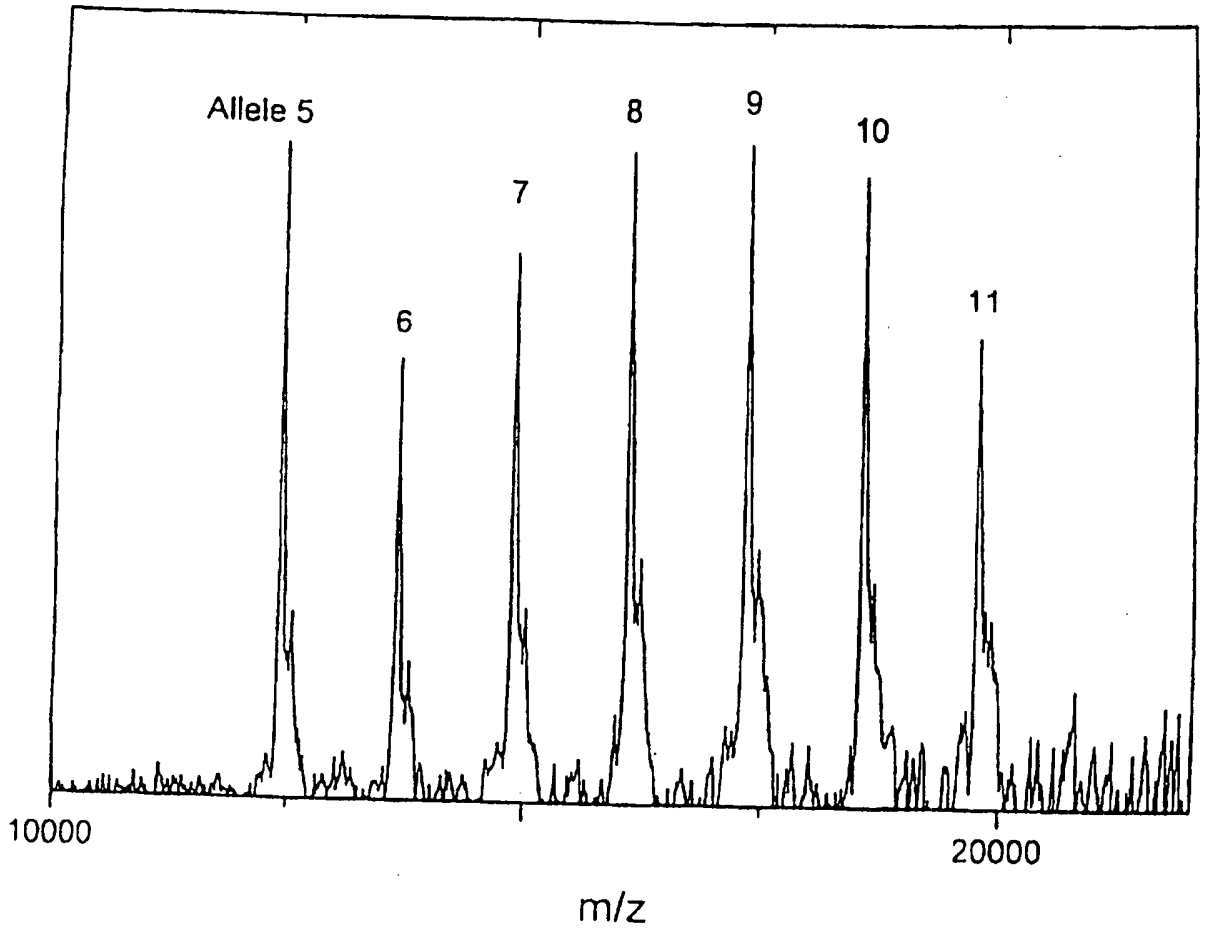

FIG. 15

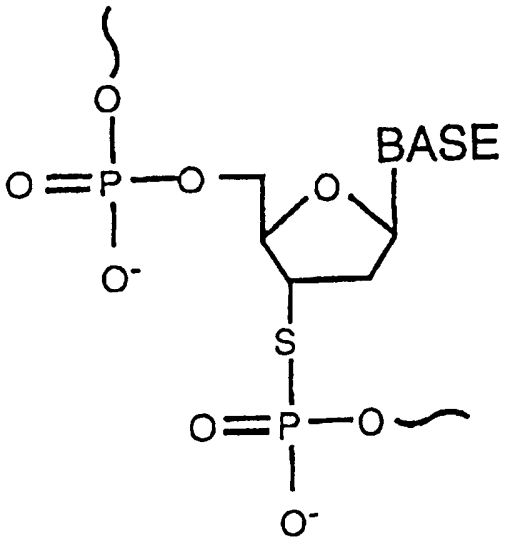

FIG. 16A

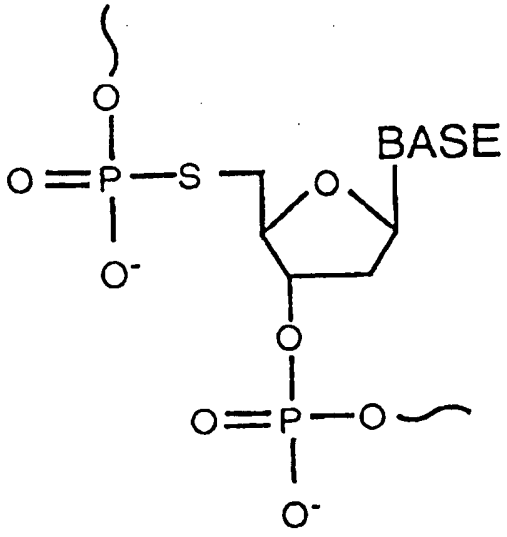

FIG. 16B

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 97/17101

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 //H01J49/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TANG K ET AL: "DETECTION OF 500-NUCLEOTIDE DNA BY LASER DESORPTION MASS SPECTROMETRY" RAPID COMMUNICATIONS IN MASS SPECTROMETRY, vol. 8, no. 9, September 1994, pages 727-730, XP000608100 see the whole document ---	1-94
Y	WO 94 16090 A (MOLECULAR TOOL INC ;NIKIFOROV THEO (US); KNAPP MICHAEL R (US)) 21 July 1994 see abstract and claims ---	1-94
A	WO 94 16101 A (KOESTER HUBERT) 21 July 1994 see abstract and claims --- -/--	10-15, 22-27, 46,47

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 January 1998	20/01/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 97/17101

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 93 08305 A (HOLMES MICHAEL JOHN ;DYNAL AS (GB)) 29 April 1993 see claims and figure	16,28, 35,48, 52,55, 69,73, 79,92
A	SZYBALSKI W: "UNIVERSAL RESTRICTION ENDONUCLEASES: DESIGNING NOVEL CLEAVAGE SPECIFICITIES BY COMBINING ADAPTER OLIGODEOXYNUCLEOTIDE AND ENZYME MOIETIES" GENE, vol. 40, 1 January 1985, pages 169-173, XP002012188 see the whole document	1-7, 17-21, 36-41, 56-62, 77,78,90
A	WU K J ET AL: "TIME-OF-FLIGHT MASS SPECTROMETRY OF UNDERIVATIZED SINGLE-STRANDED DNA OLIGOMERS BY MATRIX-ASSISTED LASER DESORPTION" ANALYTICAL CHEMISTRY, vol. 66, no. 10, 15 May 1994, pages 1637-1645, XP000579973 see the whole document	1-94
P,X	WO 97 33000 A (GENETRACE SYSTEMS INC) 12 September 1997 see the whole document	1-94

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WO 9416101 A	21-07-94	AU 5992994 A	15-08-94
		CA 2153387 A	21-07-94
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		US 5547835 A	20-08-96
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